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Onyedikachi Cecil Azuama

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Normandie Université

THESE

Pour obtenir le diplôme de doctorat

Spécialité : Aspects moléculaires et cellulaires de la Biologie)

Préparée au sein de l'Université de Rouen Normandie

Recherche de nouveaux actifs d'origine végétale contre le pathogène opportuniste de l'Homme *Pseudomonas aeruginosa*

**Présentée et soutenue par
Onyedikachi Cecil AZUAMA**

**Thèse soutenue publiquement le 18 décembre 2020
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PREAMBLE

Bacterial resistance to antibiotics is one of the biggest problems facing medicine in the 21st century. Today, the worrying expansion of this phenomenon results in more than 700,000 deaths per year, a figure which could reach 10 million in 2050 if nothing is done. Beyond this tragic observation, the economic cost of this threat is real and has been estimated at over 100,000 billion dollars for the next 30 years (O'Neill, 2016). The World Health Organization has taken up this issue and in 2015 adopted a global action plan to combat resistance. It also pre-alerted the public authorities in 2017 by publishing a list of priority pathogens most threatening to human health, in order to try to promote research and development of new antibacterials to fight them (<https://www.who.int/news-room/detail/02-27-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>). Indeed, France remains a country over-consumer of antibiotics and appears to be one of the European countries most affected by antibiotic resistance. Multi-resistance to antibiotics is therefore a major public health problem. In addition, if the existing antibiotics lose their effect, the weak investments of the pharmaceutical sector, for several decades in this field, mean that a single family of antibacterials has been discovered in the last thirty years, effective only against Gram-positive bacteria. Thus, the scientific community finds itself in an emergency situation and is confronted with the need to identify new antibacterial agents capable of combating resistant pathogens and / or of developing new strategies to combat these germs.

INTRODUCTION

PART I. *Pseudomonas aeruginosa*

1. An opportunistic pathogenic bacterium with high adaptive potential

Pseudomonas aeruginosa is a strictly aerobic γ -proteobacterium, motile by a polar flagellum and with a large genome (6.3 Mbp). This Gram-negative, mesophilic bacillus is able to adapt to many environments such as water, soil and air, thanks to a versatile metabolism and sophisticated regulation of its gene expression (**Figure 1**, Pirnay *et al.*, 2009; Selezska *et al.*, 2012).

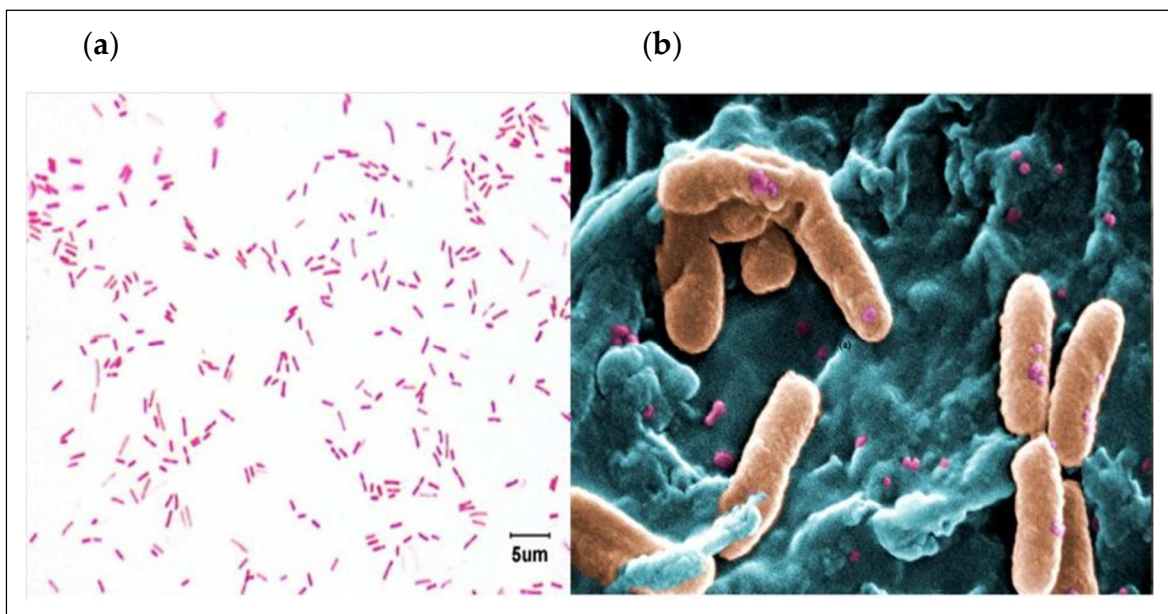


Figure 1. Different views of *Pseudomonas aeruginosa*. (a) Gram stain of *P. aeruginosa*, (b) scanning electron micrograph (SEM) of *P. aeruginosa*. Adapted from (Kaiser, 2009; Carr, 2006)

Considered as ubiquitous, this microorganism is however rarely isolated from pristine environments, suggesting that this bacterium is found in environments associated with human activity (Crone *et al.*, 2020). Its adaptive capacities allow it to respond quickly to environmental changes, which are responsible for its development in a planktonic or community lifestyles that are associated to acute or chronic infections, respectively (**Figure 1**). This bacterium can indeed cause a wide range of serious infections of the respiratory, urinary tracts, skin, ear, eye, neuro-meningea (Oliveira *et al.*, 2020). *P. aeruginosa* is also found in the tracheobronchial tree of patients with cystic fibrosis, responsible for the worsening of the symptoms linked to this disease (Lyczak *et al.*, 2000; Haussler, 2004; Starkey *et al.*, 2009).

In France and throughout the world, this bacterium represents therefore a major public health issue: it represents the 2nd cause of nosocomial pneumonia and is the 3rd most frequently identified Gram-negative bacillus species during bacteremia (Pachori *et al.*, 2019). It is responsible for infections in 60 to 80% of patients with cystic fibrosis (Acosta *et al.*, 2020). The prevalence of *P. aeruginosa* infections in the intensive care unit is of 15.3% (Hoang *et al.*, 2018). This opportunistic pathogen is indeed difficult to control in weakened or immunosuppressed people because it is inherently resistant to many antibiotics and antiseptics, thanks to an arsenal of degradation enzymes, efflux pumps, and an ability to form a resistant biofilm. This germ is also capable of rapidly acquiring new resistance under selection pressure (Breidenstein *et al.*, 2011; Poole, 2011). This combination of factors has led the World Health Organization (WHO) to classify *P. aeruginosa* as a critical priority in its list of pathogens against which research and development of new therapies must be accelerated (WHO, 2017). Almost 14% of *P. aeruginosa* isolates were classified MDR (multidrug-resistant) in 2015 (Antimicrobial resistance surveillance in Europe, European Center for Disease Prevention and Control, 2015).

2. Acute and chronic infections

To initiate infection, *P. aeruginosa* generally takes advantage of impaired immune defense mechanisms (Van Delden and Iglewski, 1998). Once in the site of infection, *P. aeruginosa* deploys its "molecular arsenal", including multiple cellular and extracellular virulence factors that allow it to develop infection and colonize the various tissues of the host (Jimenez *et al.*, 2012). These virulence factors are expressed sequentially during the different stages of host infection (Van Delden and Iglewski, 1998). The first step of the infectious process consists of adhesion to the altered epithelium by expression of cellular virulence factors, including flagella, type IV pili or lipopolysaccharide (LPS). After colonization, the infectious process evolves, either towards a chronic phase of infection characterized by the formation of biofilms, or towards an acute phase of infection, characterized by the production of extracellular virulence factors (exotoxins, exoproteases, toxins, rhamnolipids, phenazines) dependent on cell signaling, which severely damage the infected tissues and facilitate blood system invasion and bacterial spread (Van Delden and Iglewski, 1998).

Infections due to *P. aeruginosa* are thus divided into two groups, acute and chronic infections, and depend on the production of different virulence factors whose expression is linked to complex perception and communication systems (**Figure 2**, Gooderham and Hancock, 2009).

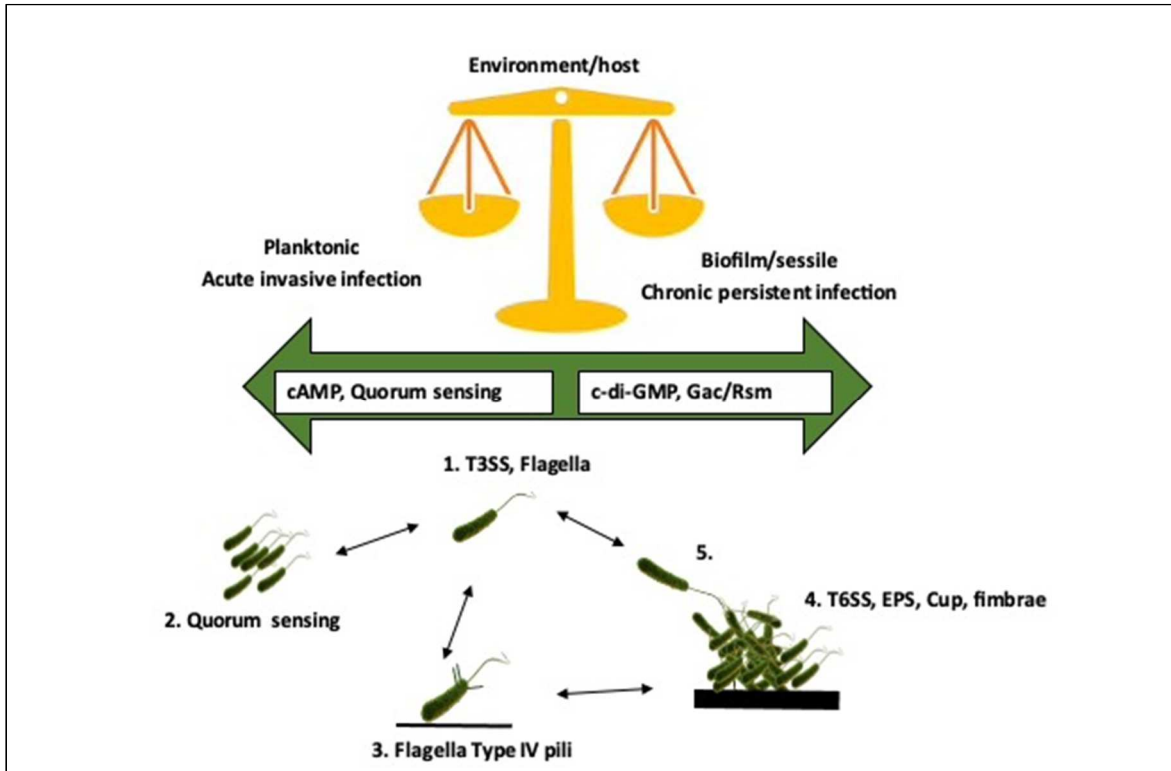


Figure 2. Adaptation of *P. aeruginosa* to two different lifestyles in host and environment. 1. *P. aeruginosa* can exist in a free-living form 2. As population density increases, they communicate using quorum sensing molecules. Upon interaction with solid surface, attachment is made via flagella and type IV pili. Once intimate surface is generated with sufficient nutrient, 4 *P. aeruginosa* forms biofilms in an enclosed matrix. 5. Unknown signals or stochastic process disperse biofilms and return to planktonic form.

* Acute *P. aeruginosa* infections are invasive and cytotoxic infections that rely on the production of:

i) factors associated with cells such as lectins, which inhibit ciliary beat of lung cells,

ii) factors secreted by secretion systems such as toxins and hydrolytic enzymes leading to an inflammatory response and necrosis of infected tissues, which can lead to sepsis,

iii) exocellular factors, which remain associated with cells while being extracellular, such as pyocyanin, a blue-green tricyclic phenazine which possesses pro-inflammatory and cytotoxic effects as an oxidizing agent; pyoverdine, a major siderophore with a strong affinity for ferric iron; or the biosurfactant rhamnolipids that are able to insert into the epithelial cells membranes.

Most of these virulence factors are dependent on the bacterial communication system called Quorum Sensing (QS) considered to be one of the main mechanisms regulating the expression of virulence genes (Ben Haj Khalifa *et al.*, 2011).

* In chronic infections, bacteria are organized into a complex bacterial community, the biofilm. In this structure, the cells are embedded in an extracellular matrix consisting of polysaccharides, rhamnolipids, proteins, nucleic acids and vesicles, giving them protection against the host immune system, antimicrobial agents and therapeutic compounds (Hancock, 1998; Ciofu *et al.*, 2012). In addition to the production of structural matrix elements, biofilm formation is associated with the expression of adhesion and mobility factors, such as lectins and type IV pili (Davey *et al.*, 2003), some of which are also under the control of QS. At the molecular level, this mode of development is also often correlated with a high intracellular level of c-di-GMP and low of cAMP (Valentini *et al.*, 2018).

This remarkable ability of *P. aeruginosa* to adapt its behavior according to its microenvironment is therefore a determining factor of its pathogenicity, which is highly and finely controlled by interconnected regulatory systems. The pathogenesis of this organism does not depend only on a single virulence factor, but on a precise interaction between various factors, which will be described below. We will therefore describe the virulence factors of *P. aeruginosa* in terms of acute or chronic virulence, and the main mechanisms regulating their production.

3. Virulence factors

Virulence factors are components associated with cells, exocellular or secreted by various secretion systems (Type I, Type II, Type III, Type V and Type VI), which are essential for the survival, colonization and invasion of host tissues by *P. aeruginosa* (**Figure 3**). In this part, the main virulence factors that contribute to its pathogenesis will be presented.

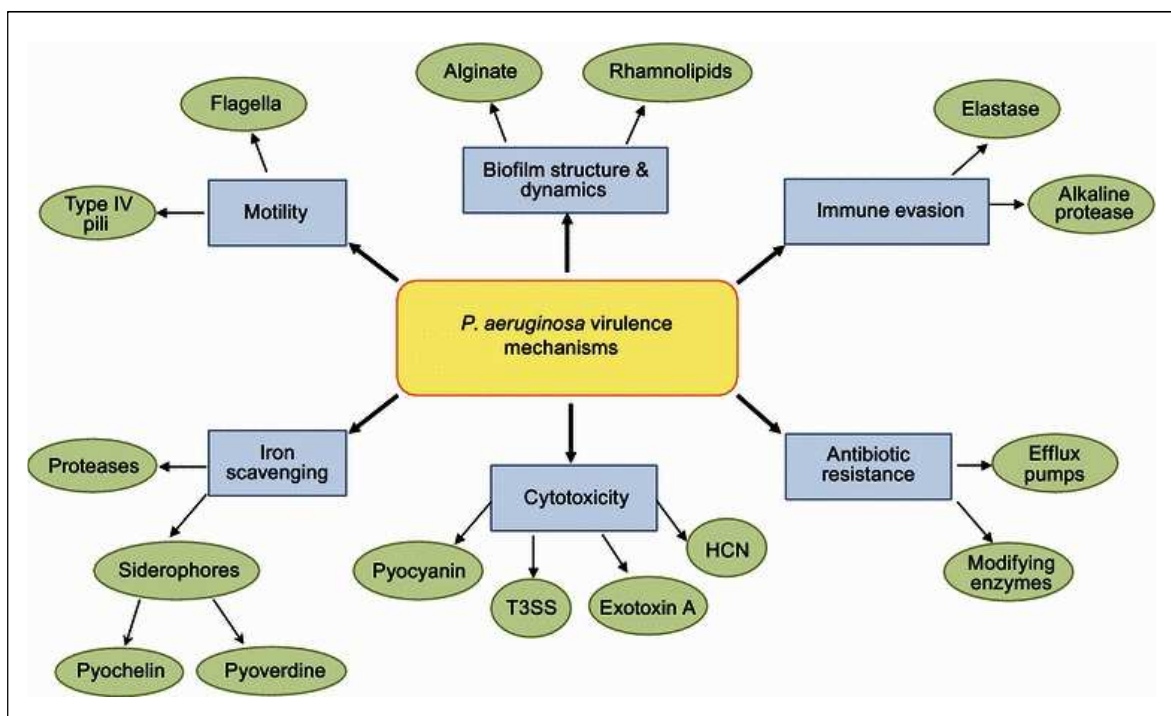


Figure 3. Several virulence factors elaborated by *P. aeruginosa* (Adapted from Lee and Zhang, 2014).

3.1. Cell-associated virulence factors

The first step in the infectious process is the colonization of the epithelial surface of the host cells. This adhesion step is achieved using adhesins such as type IV pili or fimbria, lipopolysaccharide (LPS) or flagella.

PA-IL & PA-IIL lectins

PA-IL and PA-IIL lectins are virulence factors synthesized by *lecA* and *lecB*, respectively. They are adhesins (Chemani *et al.*, 2009) showing strong affinities for galactose and fucose, respectively, sugars that can be found on the surface of both prokaryotic and eukaryotic cells (Vallet *et al.*, 2001). The PA-IL lectin plays a role in membrane permeabilization of eukaryotic cells (Laughlin *et al.*, 2000) and is toxic to respiratory cells (Bajolet-Laudinat *et al.*, 1994). The PA-IIL lectin inhibits the ciliary beat of lung cells (Adam *et al.*, 1997). The two lectins play an important role in adhesion (Chemani *et al.*, 2009) and biofilm structuration, by linking the bacteria to each other's and to the matrix exopolysaccharides (Diggle *et al.*, 2006; Passos da Silva *et al.*, 2019).

Flagella and pili

Flagella and type IV pili are involved in motility in *P. aeruginosa*. Swimming-type motility corresponds to swimming in a liquid medium and is mediated by flagella. Swarming occurs in a semi-solid medium and involves 3 major partners, flagella, type IV pili and rhamnolipids surfactants. Finally, twitching-type mobility corresponds to bacterial movement on a solid surface and is linked to type IV pili.

Flagella are involved in the pathogenesis of bacteria since these organelles not only contribute to the spread of the bacteria, but they also promote invasion and colonization of the host by acting as adhesins. Thus, the flagellum of *P. aeruginosa* has been observed to mediate adhesion to respiratory epithelial cells and to participate in biofilm formation *in vitro* (Feldman *et al.*, 1998; O'Toole and Kolter, 1998). A *fliC* mutant, which does not produce a flagellum, exhibits attenuated virulence (Kuang *et al.*, 2011). The flagellum is also involved in the formation and dispersion of the biofilm (Sauer *et al.*, 2002). Type IV pilus is the main adhesin of *P. aeruginosa* (Deziel *et al.*, 2001). Anchored in the inner membrane and emerging from

the outer membrane through the periplasm, it is made of pilin monomers. The retraction capacity of the pilus confers the bacteria twitching motility (Whitchurch *et al.*, 1991) and is also involved in the swarming motility (Kohler *et al.*, 2000). Several studies carried out *in vitro* and *in vivo* on animal models have revealed that pili are structures essential for the adhesion and invasion of *P. aeruginosa*, confirming their pro-virulent character (Comolli *et al.*, 1999). A *pilA* mutant is deficient in the formation of the typical mushroom pillars structures of the biofilm, demonstrating the importance of pili in biofilm formation (Barken *et al.*, 2008), and exhibits attenuated virulence (Comolli *et al.*, 1999).

Lipopolysaccharide

The lipopolysaccharide (LPS) is a lipoglycan anchored in the outer leaflet of the outer membrane of all Gram-negative bacilli. Besides its stabilizing action on the membrane, LPS plays a role in the pathogenicity of *Pseudomonas* and has antigenic properties. The hydrophobic domain of LPS, lipid A, is responsible for the endotoxic activity of this bacterium. LPS can trigger the innate immune response via Toll-like 4 receptors (TLR4), expressed by many immune cells such as monocytes, macrophages, dendritic cells, and neutrophils. Activation of the innate complement-mediated immune response results in an exacerbated response with the production of cytokines, chemokines, and interferons (Oliveira & Reygaert 2020). The large variability in the composition, size, and structure of the O antigen, as well as the composition and degree of acylation of lipid A strongly modulate the virulence of bacterial species and strains (Lerouge and Vanderleyden, 2002). The variability of LPS synthesized by a bacterial strain alters the host's immune response. Indeed, in *P. aeruginosa*, depending on the presence and / or the nature of the O antigen, the strains are more or less resistant to phagocytosis directed by complement (Liang-Takasaki *et al.*, 1982; Liang-Takasaki *et al.*, 1983).

3.2 Exocellular virulence factors

After this colonization stage, the infectious process evolves, either towards a chronic phase of infection characterized by the formation of biofilms, or towards an acute phase of infection, characterized by a massive production of extracellular virulence factors (exotoxins, exoproteases, toxins) dependent on QS cell signaling or as a response to iron limitation, which severely damage infected tissues and facilitate bloodstream invasion and bacterial spread (Van Delden and Iglewski, 1998).

Phenazines and pyocyanine

Phenazines are nitrogenous heterocyclic molecules that are toxic to both eukaryotic and prokaryotic cells. Although the central structure of phenazine is similar in different phenazines, the nature and position of the substituents on the heterocyclic ring primarily determine their color and biological properties (Price-Whelan *et al.*, 2006, **Figure 4**). Two groups of core phenazine biosynthetic genes can be found in the genome of *P. aeruginosa*, *phz1* and *phz2* (Mavrodi *et al.*, 2001). The products of the *phzA-G* genes are involved in the biosynthesis of phenazine-1-carboxylic acid (PCA) from chorismic acid. PCA can then be converted into phenazine-1-carboxamide (PCN), 1-hydroxyphenazine (1-HP) and 5-methylphenazine-1-carboxylic acid betaine (MPCAB) by the action of the enzymes PhzH, PhzS and PhzM, respectively. Additionally, MPCAB can be converted to pyocyanin via PhzS (Mavrodi *et al.*, 2001).

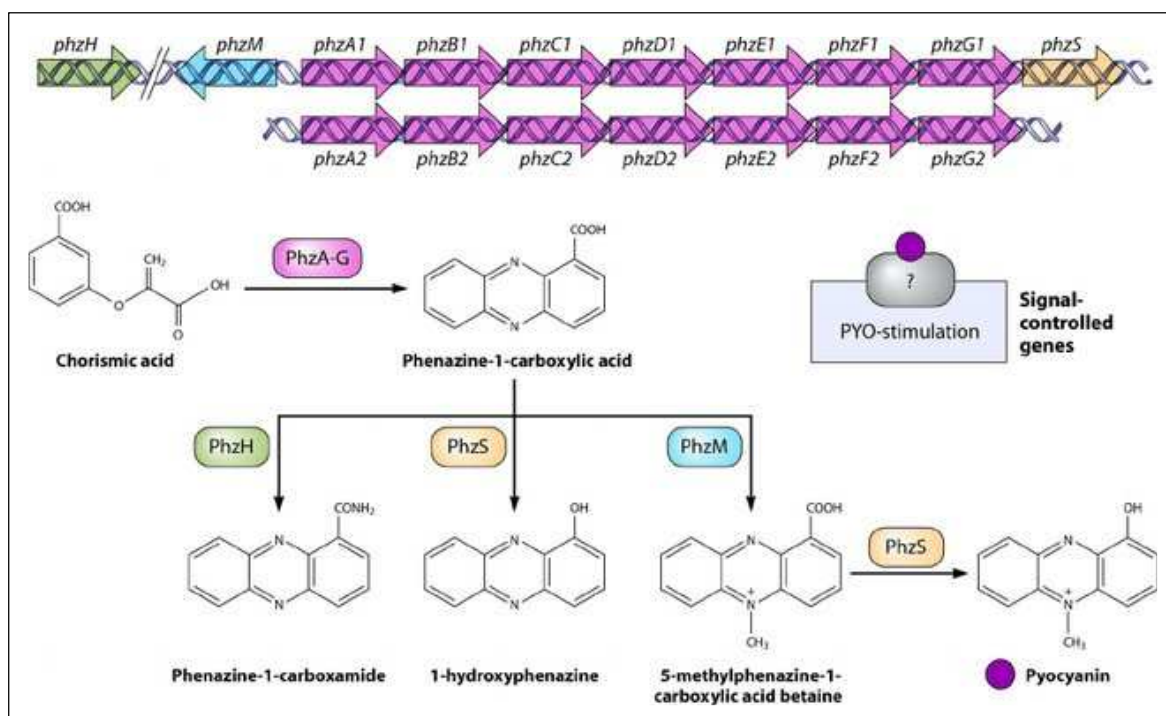


Figure 4. Steps involved in biosynthesis and signaling system of pyocyanin. PhzA to -G proteins transform chorismic acid to phenazine-1-carboxylic acid, which is subsequently converted into various phenazines by PhzH, PhzS, and PhzM enzymes. The latter product is further transformed by PhzS into pyocyanin (PYO). Next to its role as a virulence factor, PYO serves as a signaling molecule that activates a limited set of genes known as the PYO stimulon. (Jimenez *et al.*, 2012).

Pyocyanin is a blue redox-reactive compound and the most studied phenazine metabolite commonly considered as a marker of quorum sensing response (Tomas *et al.*, 2018; Vilaplana and Marco, 2020). NAD(P)H is known to reduce pyocyanin pigment which subsequently reacts with molecular oxygen to yield superoxide and hydrogen peroxide (H_2O_2), thereby causing oxidative damage to host cells (Muller *et al.*, 2002). It plays a role in acquisition of iron (Cornelis and Dingemans, 2013) and is also involved in immune response suppression and apoptosis of neutrophil in host cell (Allen *et al.*, 2005; Kipnis *et al.*, 2006). It can also inhibit cellular respiration or the

ciliary function of lung cells (Britigan *et al.*, 1992; Britigan *et al.*, 1999). Pyocyanin can also function as a signal molecule since it controls the expression of several genes involved in drug efflux, redox processes as well as iron acquisition (Dietrich *et al.*, 2006; Jimenez *et al.*, 2012).

Rhamnolipids

Rhamnolipids are glycolipid biosurfactants, which consist of one (mono-rhamnolipid) or two rhamnose groups (di-rhamnolipid) linked together by an α -1,2-glycosidic bond, and one to three fatty acid chains (8 and 16 carbons) linked by an ester bond. Rhamnose groups are linked to fatty acids by O-glycosidic bond (**Figure 5**, Abdel-Mawgoud *et al.*, 2009). Many variations exist in the structure of rhamnolipids, depending on the number of rhamnose groups, the number and length of the fatty acid chain, or the number of unsaturated bonds in the fatty acid chain, leading to more than 60 different rhamnolipid structures. Among them, the di-rhamnolipids seem to be the most abundantly produced (Abdel-Mawgoud *et al.*, 2009). They are synthesized by the enzymes RhlA, RhlB (mono-rhamnolipid), and RhlC (di-rhamnolipid) (Zhu and Rock, 2008). Rhamnolipids are involved in the swarming-like motility of *P. aeruginosa* (Kohler *et al.*, 2000), and function as wetting agents, reducing the surface tension surrounding the bacterial colony, thereby facilitating the migration of *P. aeruginosa* cells through a semi-solid surface (Caiazza *et al.*, 2005). In addition, rhamnolipids are involved in the cell dispersion of *P. aeruginosa* biofilms (Boles *et al.*, 2005) and in the maintenance of free water channels in mature biofilms (Davey *et al.*, 2003). Finally, it has been observed that rhamnolipids are capable of lysing polynuclear neutrophils surrounding the biofilms of *P. aeruginosa* to protect them against the approach of these leukocytes (Alhede *et al.*, 2009).

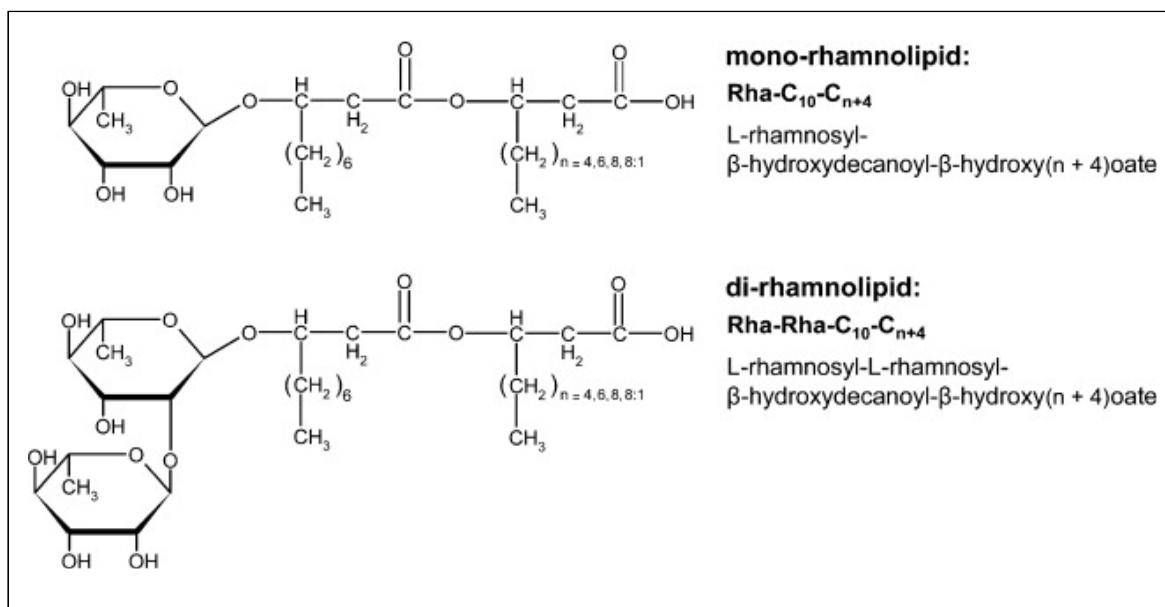


Figure 5. Structures of mono- and di-rhamnolipids.

Siderophores

Bacteria have developed different strategies to cope with iron limitation induced by the host deficiency, including the secretion of iron chelating molecules, called siderophores (Guerinot, 1994; Guerinot and Yi, 1994; Andrews *et al.*, 2003). Siderophores have a strong affinity for ferric iron, and the ferri-siderophore complexes will further be captured by external membrane receptors, which will allow their passage into the periplasm via an active TonB-dependent transport, before being translocated into the bacterial cytoplasm, where the complexed iron will be reduced to ferrous iron (**Figure 6**, Schalk *et al.*, 2002; Andrews *et al.*, 2003; Cornelis *et al.*, 2009).

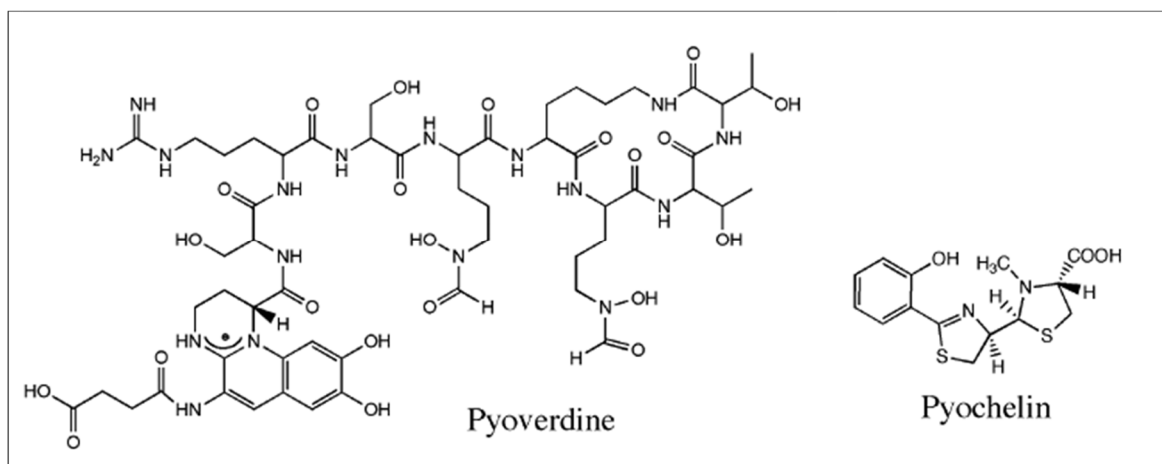


Figure 6. Structures of the pyochelin and pyoverdine siderophores

Pyoverdine, a green pigment, is the first siderophore identified in *P. aeruginosa* and chelates ferric iron Fe^{3+} at a stoichiometry of 1: 1 (Visca *et al.*, 2002). Pyocheline chelates iron at a stoichiometry of 2: 1 and has a lower affinity compared to pyoverdine because it can also complex a variety of other metals, such as aluminum, copper or even nickel (Visca *et al.*, 1992; Cunrath *et al.*, 2015). Pyoverdine biosynthesis and uptake is regulated by two sigma factors with an extracytoplasmic function (σ ECF factors), PvdS and FpvI, respectively (Chevalier *et al.*, 2019; Otero-Asman *et al.*, 2019b), the small RNAs PrrF1 and PrrF2 and the major repressor Fur (Visca *et al.*, 2002; Visca and Imperi, 2018). Pyochelin synthesis and uptake is under the control of Fur and the PchR regulator (Michel *et al.*, 2005; Cornelis *et al.*, 2009). The importance of these siderophores in bacterial toxicity has been widely demonstrated, with mutants deficient in siderophores invariably being less virulent (Meyer *et al.*, 1996; Ratledge and Dover, 2000). Pyoverdine exhibits cytotoxic activity towards human leukocytes (Becerra *et al.*, 2001). In addition to its role as an iron chelator, pyoverdine controls its synthesis in addition to that of secreted virulence factors such as endoprotease PrpL and exotoxin A (Gaines *et al.*, 2007; Chevalier *et*

al., 2019; Otero-Asman *et al.*, 2019b). Next to the two siderophores pyoverdine and pyochelin, *P. aeruginosa* has three systems to take up heme via TonB-dependent receptors and one system to take up Fe^{2+} under microaerobic conditions (Cornelis and Dingemans, 2013; Otero-Asman *et al.*, 2019a). *P. aeruginosa* has also a battery of TonB-dependent receptors recognizing other Fe-siderophore complexes coming from other microorganisms (termed xenosiderophores, Cornelis *et al.*, 2009).

3.3. Secreted virulence factors

P. aeruginosa possesses a wide variety of toxins and secreted enzymes, the secretion of which is carried out by Type 1, 2, 3, 5 and 6 secretion systems (TXSS) (Bleves *et al.*, 2010; **Figure 7**).

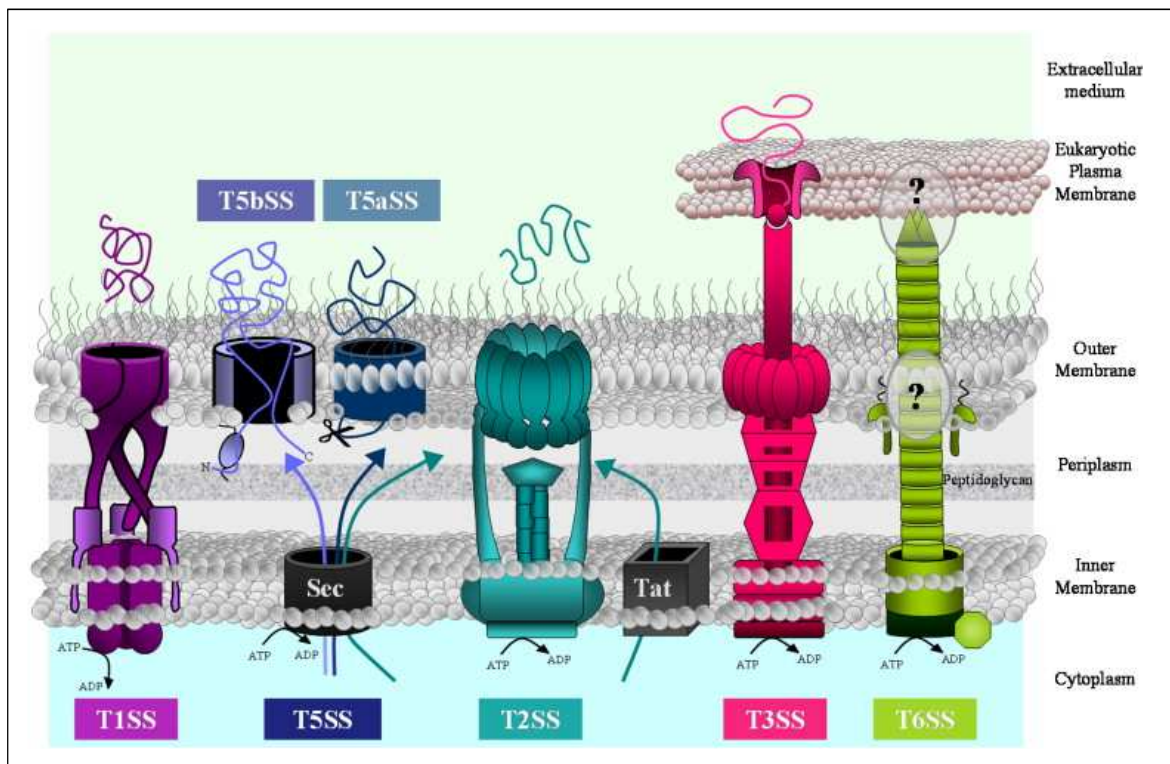


Figure 7. Schematic overview of the protein secretion systems in *P. aeruginosa* (Bleves *et al.*, 2010).

- The T1SS is an ABC-transporter, which is known to export at least 3 substrates among which the protein of unknown function AprX, the hemophore HasA for heme transport, and the alkaline metalloprotease AprA, which displays a maximal activity at alkaline pH and is capable of degrading components of the immune system such as interferons- γ , TNF- α or interleukins (Pedersen *et al.*, 1987; Theander *et al.*, 1988; Duong *et al.*, 2001; Pletzer *et al.*, 2020).

- The T2SS secretes the majority of hydrolytic enzymes and toxins, such as exotoxin A, LasA and LasB elastases or the PrpL protease (Filloux, 2011). Exotoxin A is a toxin that targets eukaryotic cells protein synthesis leading to the death of the target cell by necrosis (Wick *et al.*, 1990). Its synthesis is regulated by QS and by iron limitation via the extracytoplasmic sigma factor PvdS (Visca *et al.*, 2007; Chevalier *et al.*, 2019). Elastases LasA (also called staphylolysin) and LasB, are both able to break down elastin, with a synergistic effect. LasB is also capable of degrading collagen or to cleave type A and G immunoglobulins (Bainbridge and Fick, 1989; Heck *et al.*, 1990; Mariencheck *et al.*, 2003; Gellatly and Hancock, 2013; Thibodeau and Butterworth, 2013). These enzymes also alter tight junctions thus favoring the invasion of the eukaryotic lung epithelium (Nomura *et al.*, 2014). These proteases production is under the control of QS (Popat *et al.*, 2008). Protease IV (Piv or PrpL) is an endoprotease involved in the degradation of fibrinogen, immunoglobulin G, plasmin, plasminogen and complement (Matsumoto, 2004; Malloy *et al.*, 2005). PrpL is also involved in the cleavage of lactoferrin and transferrin, allowing iron to be released. Its biosynthesis is regulated by PvdS and QS (Chevalier *et al.*, 2019).

- The T3SS or injectisome is a system that delivers toxins (S, T, U, Y exoenzymes) directly into the cytoplasm of a eukaryotic cell after contact with the bacteria. The ExoS and ExoT exoenzymes each possess target cell GTPase inhibiting activity, as well as ADP-ribosylation activity. ExoY exoenzyme is an adenylate cyclase which

increases the level of cAMP in the target cell and ExoU exoenzyme is a phospholipase. All these toxins lead to the reorganization of the cytoskeleton and the necrosis of the target cell (Barbieri and Sun, 2004; Hauser, 2009).

- T5SS is unique in that it uses the Sec translocation machinery to transport substrates into the periplasm, and then these substrates or autotransporters are able to self-transport through the outer membrane (Pena *et al.*, 2019). The EstA autotransporter has been shown to be necessary for the production of rhamnolipids (Wilhelm *et al.*, 2007).

- The T6SS system of *P. aeruginosa* consists of three clusters H1-T6SS to H3-T6SS. These are injectisomes in which H1-T6SS targets toxins only into prokaryotic cells, while H2- and H3-T6SS target both prokaryotic and eukaryotic cells by directly injecting toxins into them (Sana *et al.*, 2016). These systems are involved in the colonization of an ecological niche and in the pathogenesis of the bacteria in the case of H2- and H3-T6SS.

4. Quorum Sensing (QS)

4.1. Generalities

P. aeruginosa is capable of controlling its virulence factor production using a unique procedure that detects its cell density and permits communication between bacteria through an intercellular signaling process referred to as quorum sensing (QS) (Smith and Iglewski, 2003; Williams and Camara, 2009). Quorum sensing was first described in *Vibrio fischeri*, a marine symbiont in which the production of bioluminescence is controlled by the *lux* system (Nealson *et al.*, 1970; Sitnikov *et al.*, 1995). QS is the key regulator of virulence factors in several pathogenic bacteria (Williams *et al.*, 2007). Approximately 5-10% of *P. aeruginosa* genes encoding proteins

involved in fundamental cellular processes or in virulence are regulated by this system (Williams and Camara, 2009; Moradali *et al.*, 2017).

QS is a cell-to-cell communication mechanism that allows bacteria to coordinate gene expression in a cell density dependent manner. This system thus allows the synchronization of the metabolism and physiology of cells in response to a given environment. Communication between bacteria is ensured by small molecules called autoinducers, which bacteria continuously produce and secrete in very small quantities. When the cell density, and therefore the concentration of these autoinducers reaches a critical threshold (the quorum), a regulatory cascade is triggered, ultimately allowing the regulation of a large number of genes in a coordinated manner, as well as a very strong increased production of autoinducers. Most of these systems self-induce once quorum is reached through a positive feedback loop (LaSarre and Federle, 2013).

In *P. aeruginosa*, three QS systems (Las, Rhl and PQS) have been described and control the expression of virulence genes in a hierarchical manner (Moradali *et al.*, 2017; Turkina and Vikstrom, 2019; **Figure 8**). The Las and Rhl systems rely on the production of *N*-acyl-homoserine lactones (AHL) as signal molecules, while the PQS system responds to 2-alkyl-4-quinolones (HAQ) (Schuster and Greenberg, 2006; Williams and Camara, 2009). At high cell density, extracellular AHLs concentrations increase and a critical intracellular threshold is reached allowing AHLs to bind to their respective LuxR-type regulator. The newly formed complex interacts with target DNA sequences and increases or blocks the transcription of certain genes, thereby regulating the expression of QS-dependent genes. The concentration of AHLs is maximum between the exponential and stationary growth phases, unlike the PQS system, which is active in the stationary phase, when the concentration of PQS released is maximum (McKnight *et al.*, 2000). However, QS is not only

dependent on the bacterial population density, but it is also induced by environmental factors (van Delden *et al.*, 2001; Williams and Camara, 2009).

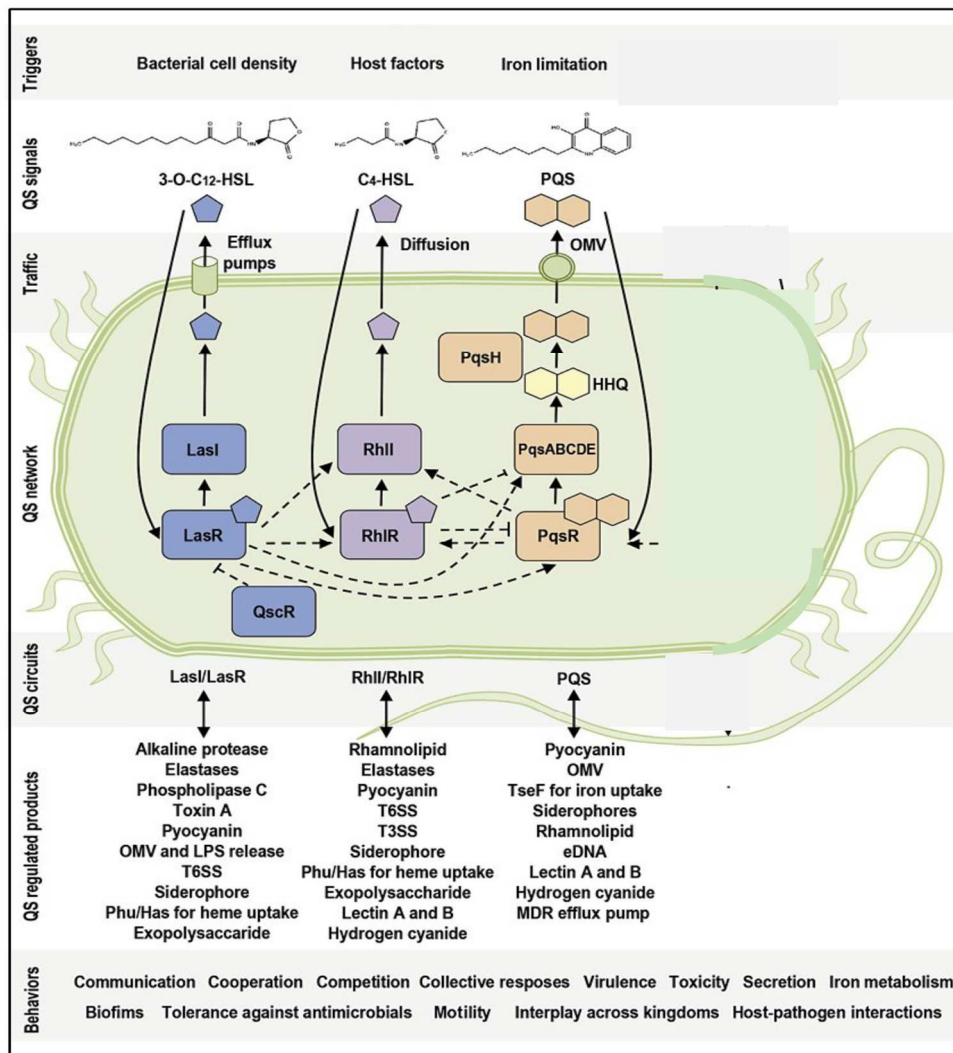


Figure 8. QS system in *P. aeruginosa*. The diagram illustrates two major circuits of the QS system: Las and Rhl, which are intertwined with the PQS system. These systems can be triggered by several stimuli, like bacteria population density, host factors, iron limitation, etc. LasI and RhII synthases synthesize the autoinducer signaling molecules 3-oxo-C₁₂-HSL and C₄-HSL respectively. These autoinducer molecules diffuse out into surrounding environment. Upon reaching a certain threshold, they activate receptors LasR and RhIR. Which control a regulon of genes including virulence factors. Modified from (Turkina and Vikström, 2019).

4.2. *Las* and *Rhl* QS systems

AHLs are composed of fatty acids the size of which is between 4 and 18 carbon atoms, linked by a peptide bond to a homoserine lactone (**Figure 8**). The fatty acid part can be modified via a 3-oxo or 3-hydroxy substituent (Hirakawa and Tomita, 2013). AHLs are synthesized by enzymes of the LuxI family, while being detected by transcriptional regulators of the LuxR family (Fuqua *et al.*, 1996). The *Las* system produces and responds to 3-oxo-C₁₂-HSL (*N*- (3-oxo-dodecanoyl) -L-homoserine lactone), while the *Rhl* system produces and is activated by C₄-HSL (*N*-butyryl -L-homoserine lactone). When a population of *P. aeruginosa* reaches a certain density at which the threshold concentration of a certain AHL is reached, AHL will form a complex with a LuxR regulator, thus activating the transcription of a number of virulence genes, as well as its own gene and that of the corresponding *luxI* gene (Pesci *et al.*, 1997; Schuster and Greenberg, 2006; Turkina and Vikstrom, 2019). The efflux of 3-oxo-C₁₂-HSL to the external environment could involve the MexAB / OprM efflux pump (Evans *et al.*, 1998; Pearson *et al.*, 1999), although the involvement of this pump is controversial (Alcalde-Rico *et al.*, 2020). Radiolabeling studies revealed that C₄-HSL diffused freely through the membrane, unlike 3oxoC₁₂-HSL (Pearson *et al.*, 1999). The main virulence factors controlled by the *Las* and *Rhl* systems are shown in **Figure 8**.

4.3. *PQS* QS system

The third communication system is based on the use of an alkyl quinolone as a communication molecule, 2-heptyl-3-hydroxy-4-quinolone, also known as Pseudomonas Quinolone Signal (PQS, **Figure 9**). The transcriptional regulator PqsR (MvfR) controls the expression of the *pqsABCDE* and *phnAB* operons encoding the enzymes involved in the biosynthesis of signaling molecules which belong to the

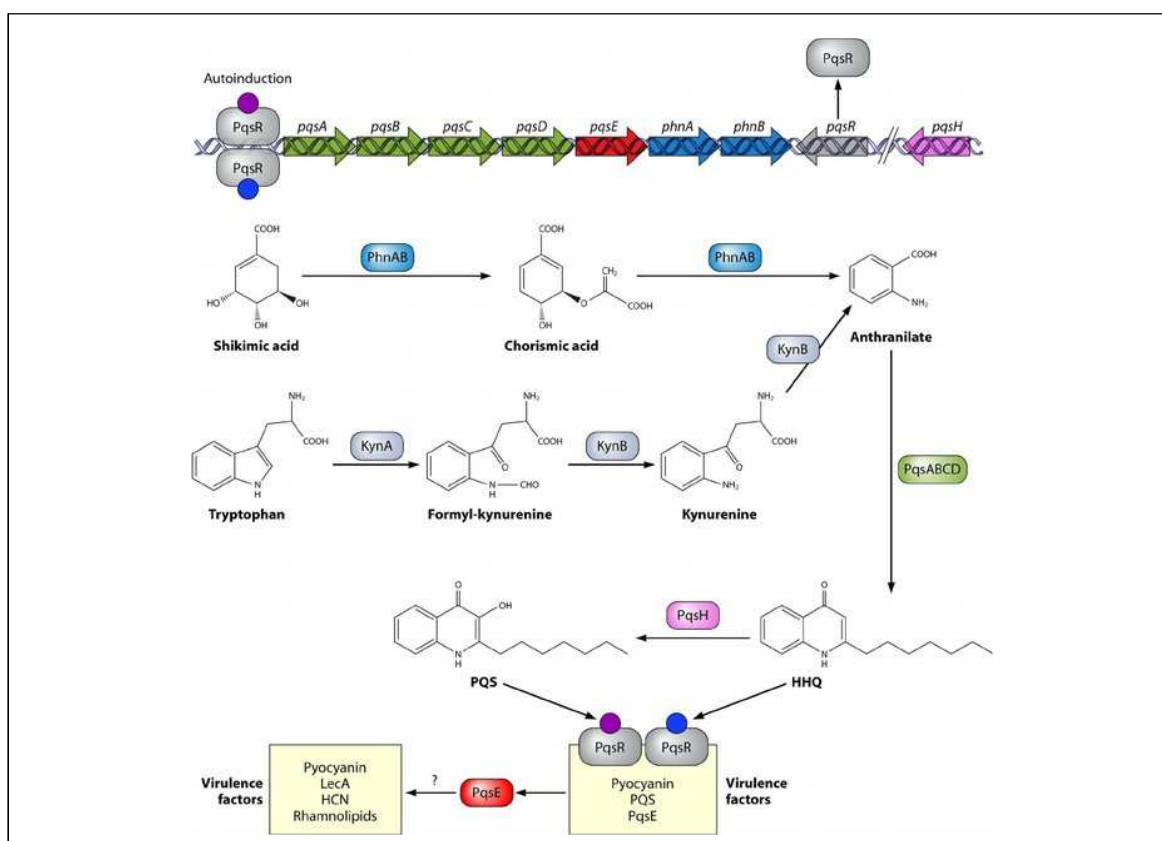


Figure 9. Biosynthesis, autoinduction, and virulence regulation by 4-alkylquinolones in *P. aeruginosa*. The synthesis of PQS begins with the conversion of anthranilate (by the PqsABCD proteins) (which originates from either the kynurenine pathway or the PhnAB anthranilate synthase) into HHQ, which is finally converted into PQS by the PqsH monooxygenase. Both HHQ and PQS bind the PqsR regulator, forming a complex that activates the transcription of the *pqsABCDE* and *phnAB* operons, leading to increased levels of PQS (autoinduction) and pyocyanin production. In addition, transcription of the PQS operon results in an increase in the levels of PqsE, an enzyme with unspecified function that increases the levels of pyocyanin, lectin, HCN, and rhamnolipids (Jimenez *et al.*, 2012).

family of 4-hydroxy-2-alkylquinolines (HAQ). The *phnAB* operon encodes the α and β subunits of the anthranilate synthase PhnAB, responsible at least partly for the biosynthesis of anthranilic acid from chorismic acid (Gallagher *et al.*, 2002). The tryptophan kynurenin degradation pathway also allows the production of

anthranilic acid (Farrow and Pesci, 2007). The *pqsABCDE* operon encodes the enzymes PqsA, PqsB, PqsC, PqsD and PqsE which are necessary for the biosynthesis of HAQ, with the exception of PqsE. PqsA, PqsB, PqsC, PqsD catalyze the conversion of anthranilate to 2-heptyl-4-quinolone (HHQ). HHQ is then hydroxylated with PqsH, allowing PQS to be produced. This molecule binds to PqsR (or MvfR) to activate the transcription of genes under its dependence (Diggle *et al.*, 2007). PqsE appears to be involved in the regulation of the expression of many virulence factors controlled by the PqsR regulon, such as pyocyanin, rhamnolipids, lectins, and elastases (Gallagher *et al.*, 2002; Diggle *et al.*, 2003; Rampioni *et al.*, 2016; Schutz and Empting, 2018). HHQ also plays a signaling role (Diggle *et al.*, 2007; Kim *et al.*, 2010). This system is most often presented as a modulator of the other two QS systems, because, in addition to self-regulating and regulating the *pqsABCDE* operon, PqsR is also involved in the regulation of *lasR* and *rhlR* (Maura *et al.*, 2016). PQS and PqsR regulate up to 140 genes, the majority of which are co-regulated by Rhl (Deziel *et al.*, 2005). On the basis of pathogenicity traits, PQS is involved in the regulation of genes responsible for pyocyanin, hydrogen cyanide, lectin, rhamnolipids, siderophore and biofilm synthesis among others (Rampioni *et al.*, 2016; Schutz and Empting, 2018). More than 50 molecules belonging to the HAQ family have been identified in *P. aeruginosa* (Leisinger and Margraff, 1979; Lepine *et al.*, 2004). The most abundant HAQs produced by *P. aeruginosa* are those which carry a seven-carbon aliphatic chain, namely HHQ (4-hydroxy-2-heptylquinoline), PQS (3,4-dihydroxy-2-heptylquinoline, or Pseudomonas Quinolone Signal) and HQNO (4-hydroxy-2-heptylquinoline N-oxide). However, only HHQ and PQS are known to be self-inducing and therefore considered to be signal molecules among all the quinolones produced by *P. aeruginosa* (Diggle *et al.*, 2011).

PQS is a relatively hydrophobic molecule, very poorly soluble in aqueous solvents (Calfee *et al.*, 2005) and possessing a high affinity for membranes rich in

lipids (Lepine *et al.*, 2003). This suggests that *P. aeruginosa* has developed a means to solubilize this molecule. Rhamnolipids contribute to the solubilization of PQS and increase the uptake of PQS in cells (Calfee *et al.*, 2005). But PQS is predominantly found in membrane vesicles which can contain up to 86% of the total PQS production by *P. aeruginosa* (Mashburn and Whiteley, 2005), and PQS is also involved in vesicle biogenesis (Cooke *et al.*, 2019). PQS has also been reported to form complexes with iron (III), indicating that in addition to its function as a signal molecule, it acts as an iron chelator to accelerate iron delivery associated with siderophores while not being a siderophore (Diggle *et al.*, 2007; Maura *et al.*, 2016; Lin *et al.*, 2018).

PQS positively activates the expression of RhII synthase (McKnight *et al.*, 2000) while in turn the Rhl system negatively controls the alkyl quinolone quorum sensing system (McGrath *et al.*, 2004). Furthermore, LasR-C12-HSL positively regulates MvfR which is an indication that PQS production requires the strict balance in the ratio of LasI and RhII autoinducer molecules (McGrath *et al.*, 2004). AQ-mediated signaling plays a major role in *P. aeruginosa* QS network. PQS exhibits vital role in mice and nematode infection (Gallagher and Manoil, 2001; Lau *et al.*, 2004; Xiao *et al.*, 2006). Moreover, PQS has also been identified in the sputum and bronchoalveolar lavage from cystic fibrosis patients (Collier *et al.*, 2002).

4.4. QS regulators

The QS is itself controlled by numerous additional regulators (Coggan and Wolfgang, 2012; Asfahl and Schuster, 2017), most of which are represented in **Figure 10**. To these are also added the small RNAs PrrF1, PrrF2 and PhrS which have also been described as regulators of QS (Sonnleitner *et al.*, 2011; Rutherford and Bassler, 2012). For instance, the transcriptional regulator RsaL binds to the *lasI* promoter to

inhibit *lasI* expression (Rampioni *et al.*, 2006). LasR / C₁₂-HSL positively controls *rsaL* expression, thus creating a negative feedback loop, which counterbalances the positive feed-back loop of the Las system thereby promoting stabilized *lasI* expression and C₁₂-HSL production. Since majority of the QS-regulated genes are expressed during stationary phase, almost 40% of these genes are also modulated by the stationary phase sigma factor RpoS (Schuster *et al.*, 2004). *rpoS* expression is partially modulated by the Rhl QS-network (Latifi *et al.*, 1996; Wagner *et al.*, 2003). During stationary growth phase RpoS activates *lasR* and *rhlR* expression and participates in the restriction of the QS-regulation of a subset of genes until the commencement of stationary growth phase. Another global regulator MvaT, has been implicated to regulate the timing and magnitude of QS-dependent gene expressions (Diggle *et al.*, 2002). Other global regulators are involved in fine-tuning of *P. aeruginosa* QS-system. VqsR (virulence and quorum-sensing regulator) for instance, is known to control the production of autoinducers and virulence factors and was shown to be under the control of the las QS-system (Juhas *et al.*, 2004). On the other hand, factors involved in controlling the activation threshold of QS-controlled genes have been reported. Three anti-activator proteins, QteE, QscR, and QslA, are involved in the sequestration of QS regulators in order to increase the threshold for induction and retard expression of QS target genes (**Figure 10**) (Asfahl and Schuster, 2017). QscR, regulates the timing of QS-activation through *lasI* repression (Chugani *et al.*, 2001). Recently it has been shown that QscR impact on QS is indirect via the direct regulation of a single linked operon (Asfahl and Schuster, 2017; Ding *et al.*, 2018). Also. QteE exerts negative post-translational control of LasR and RhlR. QslA was reported to modulate LasR activity through protein-protein interaction.

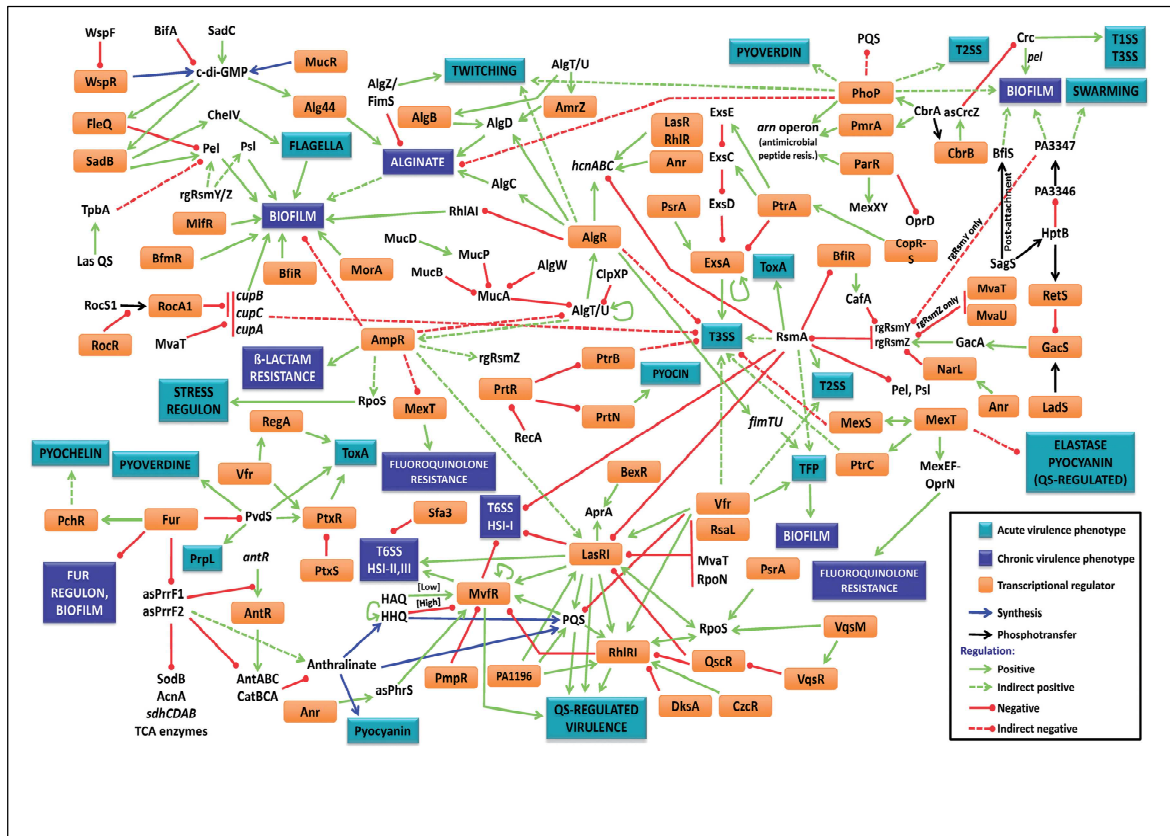


Figure 10. The *P. aeruginosa* virulence regulatory network. The pathogenic potential of *P. aeruginosa* is dictated by multiple virulence systems that are regulated transcriptionally, post-transcriptionally and post-translationally. The central mechanism for *P. aeruginosa* virulence regulation is QS, which controls expression of many virulence factors in a population density-dependent manner. Key activators of this system are LasR, RhlR, MvfR, VqsR, the cAMP receptor protein Vfr and the stationary phase sigma factor RpoS. Las system repressors include RsaL, the H-NS protein MvaT, the sigma factor RpoN and the sRNA-binding protein RsmA, whereas others like QscR repress both the Las and Rhl systems (Balasubramanian *et al.*, 2012).

Since both QslA and QscR influence DNA binding and dimerization of LasR respectively, these proteins are important for initiating the QS threshold by modulating free LasR levels. In view of the key role played by QS-communication system in the coordination of multiple *P. aeruginosa* virulence activities, the QS-regulatory network represents a potential therapeutic target.

5. Biofilms and chronic infections

When not eradicated during the colonization phase, bacteria can adapt to their environment and develop a sessile and communal way of life, the biofilm (Van Delden and Iglewski, 1998). In this form, *P. aeruginosa* adopts a less aggressive phenotype by ceasing to produce the factors associated with its acute toxicity, and protects itself against desiccation, antimicrobials and effectors of the host immune system (Reichhardt and Parsek, 2019). This biofilm induces persistent inflammation and then usually causes chronic infection, which can lead to progressive tissue destruction. Chronic *P. aeruginosa* infections are common in people with respiratory problems, such as chronic obstructive pulmonary disease and bronchiectasis, and especially in patients with cystic fibrosis (Gellatly and Hancock, 2013; Mulcahy *et al.*, 2014). Although the biofilm can form on native tissues, biofilm infections are however mostly observed on invasive medical devices such as urinary probes, osteo-articular materials or even endotracheal probes for example (Lebeaux *et al.*, 2014). Biofilms are involved in 65% of microbial infections and more than 80% of chronic infections (Jamal *et al.*, 2018).

A biofilm is a microbial community adhered to a surface or in which the germs are adhered to each other in suspension, stuck in a matrix composed mainly of exopolysaccharides, extracellular DNA (eDNA), proteins and outer membrane vesicles (Flemming and Wingender, 2010; Mulcahy *et al.*, 2014). The development of biofilms involves different stages of formation (**Figure 11**): (I) reversible attachment of the bacteria to the host surface; (II) irreversible attachment to surfaces and development into micro-colony (III); differentiation of the biofilm and development of its architecture; (IV) maturation of the biofilm and (V) dispersion of

the biofilm (Stoodley *et al.*, 2002). Biofilm formation is mediated in part by QS (Davies *et al.*, 1998; Diggle *et al.*, 2006) and depends on factors such as iron concentration (Bollinger *et al.*, 2001; Banin *et al.*, 2005), and nutritional status (Sauer *et al.*, 2004). The formation and maintenance of the integrity of the biofilm architecture is provided by an exocellular matrix. It limits the rate of penetration and diffusion of certain antibiotics and creates a stratified environment in which the center exhibits little or no oxygen, and is characterized by low bacterial growth and low metabolic activity (Anwar and Costerton, 1990). Therefore, oxygen and nutrient gradients induce the development of bacterial subpopulations with varying levels of antibiotic sensitivity (Jefferson *et al.*, 2005).

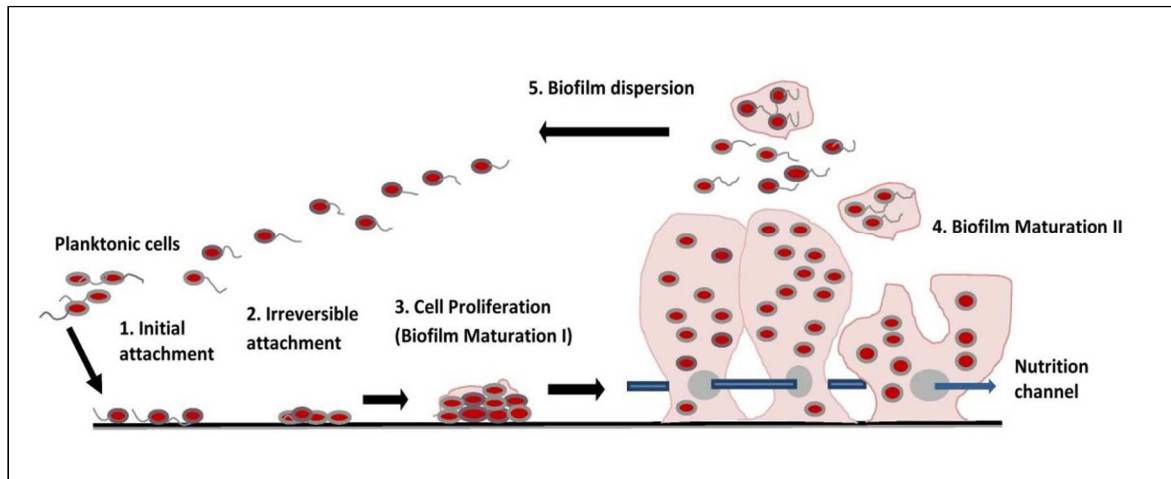


Figure 11. Various stages involved in biofilm development: 1. Initial reversible adherence of planktonic cells to surface, 2. Irreversible adherence to surface, 3. Cells proliferation and microcolony development 4. Formation maturation 5. Biofilm dispersion. Adapted from the dissertation of Pratiwi, 2015.

The extracellular matrix is mainly composed of exopolysaccharides (EPS), but also of proteins, lipids, extracellular DNA, rhamnolipids and outer membrane vesicles (Mann and Wozniak, 2012). *P. aeruginosa* produces at least three EPSs (alginate, Pel and Psl), which are critical for the structural stability of the biofilm. Pel and Psl serve as the primary scaffolding structure for biofilm development, Pel being

involved in the initiation of biofilms and Psl, in the maturation of biofilms (Ma *et al.*, 2006; Colvin *et al.*, 2011). Rather, *P. aeruginosa* secretes Psl to colonize surfaces while it has been shown to overproduce alginate during growth under certain physical conditions such as low fluid shear (Crabbe *et al.*, 2008) or following acquisition mutations during growth in the pulmonary environment of cystic fibrosis (Pedersen *et al.*, 1992). Alginates may contribute to structural stability and biofilm protection, in response to certain environmental conditions (physical stress) (Crabbe *et al.*, 2008; Crabbe *et al.*, 2010), and in patients with cystic fibrosis (Lyczak *et al.*, 2002; Feliziani *et al.*, 2010).

In fact, *P. aeruginosa* can undergo mucoid conversion following mutations in the *mucA* gene mainly, leading to hyperactivity of the extracytoplasmic sigma factor AlgU, which controls the expression of alginates (Ramsey and Wozniak, 2005; Chevalier *et al.*, 2019). These polymers of glucuronic and mannuronic acids promote irreversible attachment of the pathogen to pulmonary epithelial cells and inhibit clearance function (Ramsey and Wozniak, 2005). The extracellular appendages of *P. aeruginosa*, such as flagella, type IV pili and fimbriae, are also considered to be components of the biofilm matrix playing an adhesive role in cell-surface interactions (Ryder *et al.*, 2007; Ben Haj Khalifa *et al.*, 2011; Gellatly and Hancock, 2013; Rasamiravaka *et al.*, 2015a). In addition, rhamnolipids biosurfactants are necessary for the remodeling of the biofilm matrix (Pamp and Tolker-Nielsen, 2007) and promote the dispersal mechanisms that allow the release of planktonic cells (Boles *et al.*, 2005). The production of the exocellular matrix is partly dependent on the intracellular level of c-di-GMP, which, in high concentration promotes the production of the extracellular matrix while a low level will promote mobility and therefore planktonic lifestyle (Borlee *et al.*, 2010). An adhesin regulated by c-di-GMP, CdrA, structures the matrix by binding Psl to the bacterial membrane (Borlee *et al.*, 2010).

6. Regulation of the switch between the planktonic and sessile lifestyles

6.1. The Gac/Rsm pathway

One of the most important two-component systems for regulating the transition from acute to chronic infections is the GacS/GacA system (**Figure 12**). GacS is a sensor that will auto-phosphorylate in response to a signal that remains unknown. The phosphate group is then transmitted to GacA, which will activate the transcription of RsmY and RsmZ, two non-coding RNAs (ncRNA). These ncRNAs will sequester RsmA, a protein repressor capable of binding to messenger RNAs (mRNAs), attenuating the translational repression of many virulence factors associated with chronic infection, while repressing virulence factors associated with acute infection (Heurlier *et al.*, 2004; Kay *et al.*, 2006). Indeed, this repressor, in its free form, will directly inhibit production of functions involved in biofilm formation, such as the synthesis of EPS or the type VI secretion system (**Figure 13**). It is also capable of activating functions involved in acute virulence such as the type III secretion system, iron acquisition systems, or swarming motility via rhamnolipids and type IV pili production (Burrowes *et al.*, 2006; Brencic and Lory, 2009). The sequestration or not of this protein therefore makes it possible to orient the lifestyle of the bacteria towards the biofilm or the "free" planktonic form (Burrowes *et al.*, 2006; Brencic and Lory, 2009). Several other kinases have been shown to interact with the GacS / GacA system. LadS acts in parallel with GacS and upregulates *pel* genes involved in biofilm formation, while downregulating genes involved in the type III secretion system (Ventre *et al.*, 2006). On the opposite, RetS antagonizes GacS and activates the expression of virulence factors associated with acute infection (Ventre *et al.*, 2006). RetS can form heterodimers with GacS, preventing autophosphorylation of GacS, interfering with phosphorylation of GacA. This results in repression of

virulence factors associated with chronic infection and increased expression of virulence factors associated with acute infection (Jimenez *et al.*, 2012; Chambonnier *et al.*, 2016).

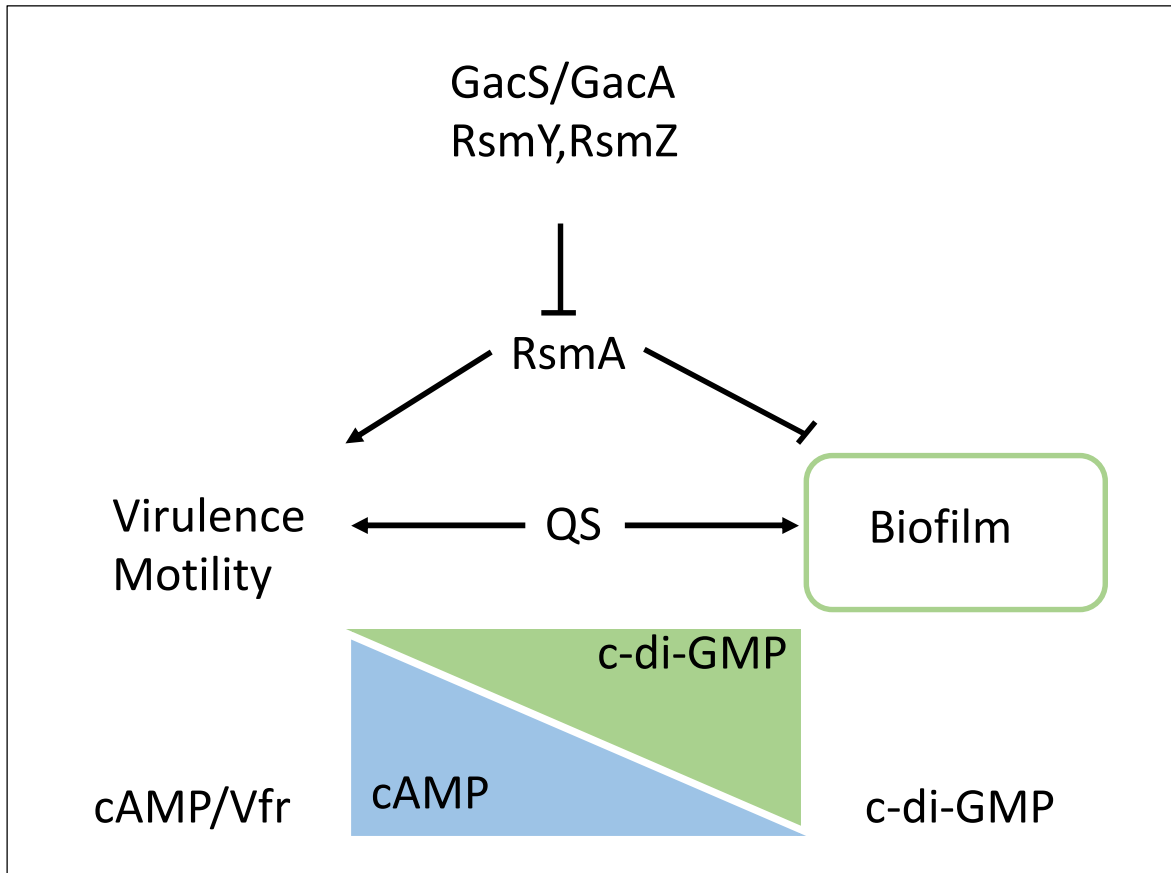


Figure 12. Representation of the global virulence regulation network of *P. aeruginosa*. Bacterial virulence and motility, mainly related to planktonic life, are activated by cAMP, QS and the post-transcriptional regulator RsmA. Biofilm lifestyle is favored by c-di-GMP, QS as well as by sequestration of RsmA by small RNAs RsmY and RsmZ regulators. Vfr has both a positive and negative effect on QS. c-di-GMP inhibits the *rhl* and *pqs* pathways as well as cAMP synthesis, while cAMP activates the degradation of c-di-GMP.

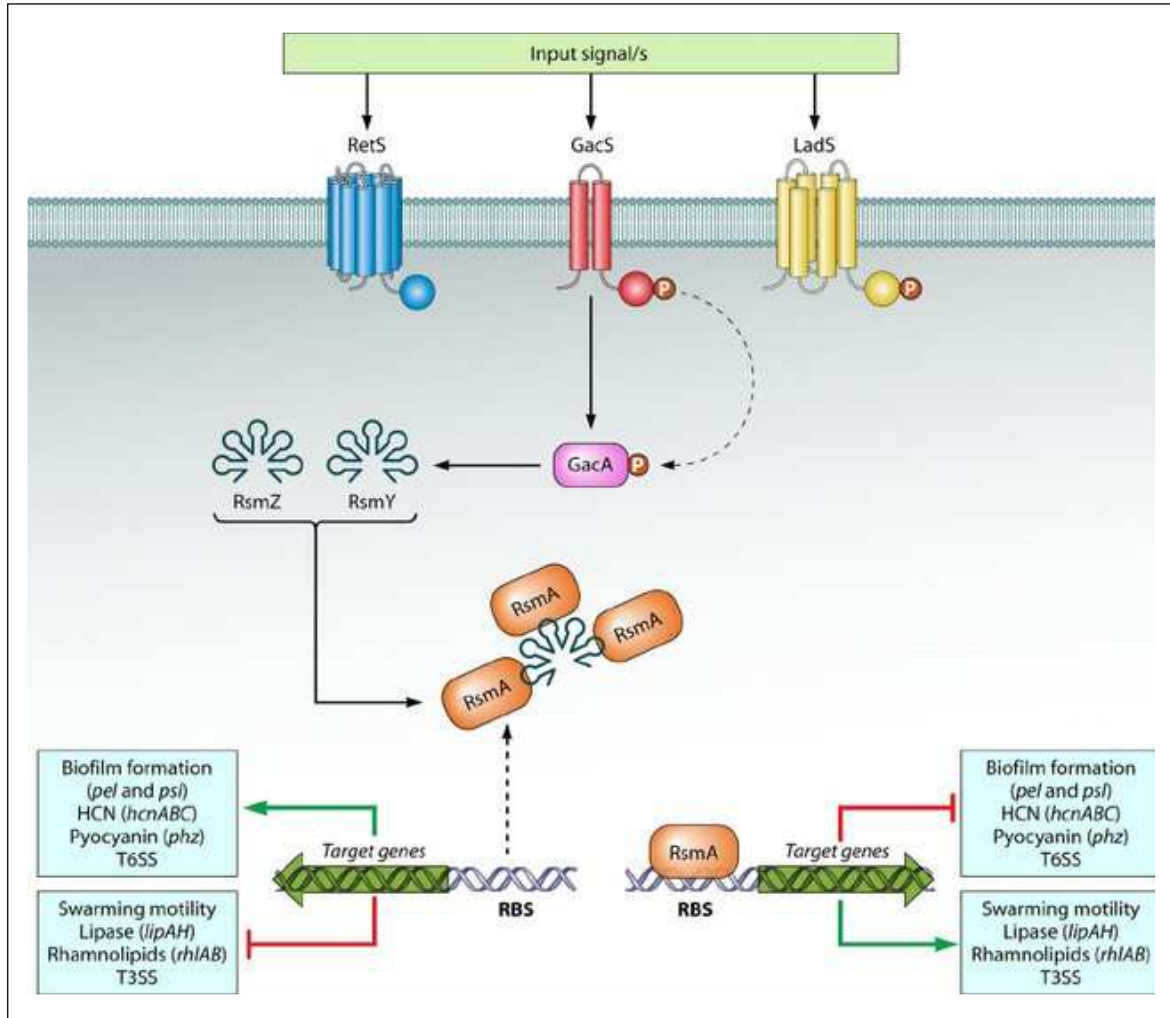


Figure 13. The Gac system network in *P. aeruginosa* controls the reversible transition from acute to chronic infections. The small regulatory protein RsmA binds to the promoters of multiple genes, enhancing bacterial motility and activating the production of several acute virulence factors while repressing the production of virulence factors associated with chronic infections. GacA phosphorylation via GacS stimulates the production of the small RNAs RsmZ and RsmY, which bind to the RsmA protein, releasing the repression of virulence factors associated with chronic infections and repressing the production of acute infection-associated factors. The sensor kinase LadS works in parallel to GacS, activating RsmZ and RsmY production, while the sensor kinase RetS acts in an opposite manner to LadS and GacS, forming a protein-protein complex with GacS that blocks RsmY and RsmZ production (Jimenez *et al.*, 2012).

6.2. cAMP and c-di-GMP second messengers signaling

cAMP (or cyclic adenosine monophosphate) is a secondary messenger produced by adenylate cyclases, which is a major mediator of virulence factors expression in case of acute infection (planktonic lifestyle). cAMP binds to the global virulence factor regulator (Vfr) inducing the production of virulence factors such as exotoxin A, T3SS toxins, and the expression of QS Las and Rhl systems (West *et al.*, 1994; Kanack *et al.*, 2006; Fuchs *et al.*, 2010). c-di-GMP (cyclic di-guanosine monophosphate) is a second intracellular messenger involved in the transition from planktonic to sessile lifestyles (Valentini and Filloux, 2016; Moradali *et al.*, 2017), and is inversely correlated with that of cAMP (Almblad *et al.*, 2015). A biofilm lifestyle will be characterized by a high level of c-di-GMP and a low level of cAMP, and vice versa for a planktonic lifestyle. c-di-GMP is synthesized from two guanosine triphosphates (GTP), by diguanylate cyclases containing a GGDEF domain), and degraded by phosphodiesterases (EAL domain). Some enzymes have both activities. In *P. aeruginosa* PAO1, there are 17 diguanylate cyclases, 5 phosphodiesterases, and 16 enzymes possessing both activities (Simm *et al.*, 2004). Interestingly, most of these enzymes contain various sensor protein domains, or belong to protein systems binding sensor proteins and those enzymes that synthesize or degrade c-di-GMP, suggesting that environmental conditions affect the level of c-di-GMP within the cell (Ha and O'Toole, 2015).

PART II. STRATEGIES TARGETING *P. aeruginosa* INFECTIONS

1. Concept of anti-virulence therapy

With the alarming increase in resistance of pathogenic bacteria to antimicrobial therapy, there has been a tremendous progress in understanding bacterial virulence strategies and the induced molecular pathways of infections, which has revealed new potentials for targeting and interfering with important pathogenic factors or traits associated with virulence in bacteria. Besides, the discovery of new antibiotic is scarce and time consuming, thereby posing a great challenge in medical therapy. Hence, novel treatments approach to *P. aeruginosa* infections are needed. In recent times, novel bacterial targets that do not affect bacterial cell growth or viability, also known as anti-virulence therapy, have been discovered. Molecules acting in this fashion are also called patho- or virulence blockers as they assuage the pressure on the pathogen to develop resistance by exclusively affecting the pathogens elaborating the targeted virulence factor (Lee and Boucher, 2015; Zambelloni *et al.*, 2015). In addition, screening assays for small-molecule inhibitors and structure-based tailoring of intervention strategies are in serious development. This concept specifically impedes with the bacteria's ability to perceive host signals that alert the pathogen of their presence at infection site and functions to attenuate specific bacterial virulence factors that are required for the establishment of infection. In addition, this strategy rather than killing the bacteria or affecting bacterial growth, presumably lowers the selection for resistance development induced by antimicrobials as most of the virulence factors are non-essential for the survival of pathogens (Rasko *et al.*, 2008; Rasko and Sperandio, 2010). It is thought that this kind

of interference will enable the clearance of pathogen by the host immune system including the prevention of pathogenic bacterial colonization by host microbiota. Moreover, these anti-virulence agents can be synergistically used in combination with conventional antimicrobials to extend the lifespan of these drugs (Cegelski *et al.*, 2008; Paul and Leibovici, 2009; Muhlen and Dersch, 2016).

2. Some concerns against virulence attenuation strategy

With the increasing literature studies and supporting evidence, plant derived anti-virulence agents have been recently considered to play great role as new drug leads in the development of effective therapeutics. Though many articles have reported promising results in the reduction of microbial pathogenicity using anti-QS approach (quorum quenching or QQ), serious concerns have been raised on the negative sides of QQ therapy, with much emphasis on the three major attributes of anti-QS molecules. The first objection is the selectivity of anti-QS molecules. It is not surprising that disrupting quorum sensing signalling may directly or indirectly affect the capacity of the human microflora to adhere and synthesize metabolites with antimicrobial activity and thus, may disturb microbiota homeostasis (Krzyzek, 2019). In addition to modulating the metabolic activity of the microbiota, quorum sensing may impact negatively on host signalling factors. For instance, AHLs are considered as neutrophil chemoattractants (Zimmermann *et al.*, 2006). In vitro and in vivo studies have illustrated their direct involvement in induction of pro-apoptotic and pro-inflammatory responses in eukaryotic cells (Grabiner *et al.*, 2014; Losa *et al.*, 2015) associated with the differentiation of fibroblasts to myofibroblasts, which important for tissue regeneration (Kanno *et al.*, 2013; Kanno *et al.*, 2016) and stimulation of protective innate immune responses (Khajanchi *et al.*, 2011).

The second objection concerning the use of anti-QS therapy raises awareness on the possibility of promoting the growth of isolates with enhanced survival ability and hence more difficult to eliminate. For instance, an *in vitro* study demonstrated that Δ lasR mutants had 4-12-fold greater pyocyanin production and 1.5-fold higher motility, but a significantly lower elastase production (Lujan *et al.*, 2007). This implies possibility of exacerbation of pro-inflammatory reactions at infection site following the use of anti-QS molecules. The final objective is the possibility of resistance development to anti-QS agents through the reduction of the levels of signalling molecules required for QS-activation (Beckmann *et al.*, 2012) or through the mutation of genes encoding efflux pumps as seen with brominated furanones (Garcia-Contreras *et al.*, 2015).

3. Main targets in *P. aeruginosa*

Many efforts have been made to develop anti-virulence agents targeting virulence factors or pathogenesis regulatory mechanisms. Some examples of potential targets shown in **Figure 14**, will be described below.

3.1. Inhibition of adherence

Bacteria can be denied access to the host by inhibiting the formation of pili or adhesins normally used for interaction and adherence to host cell receptors. The adhesion of bacteria to host surfaces facilitates initiation of infection and activation of secretion systems (Thanassi *et al.*, 2012). In addition, it enables the pathogen to overcome immunological and mechanical clearance.

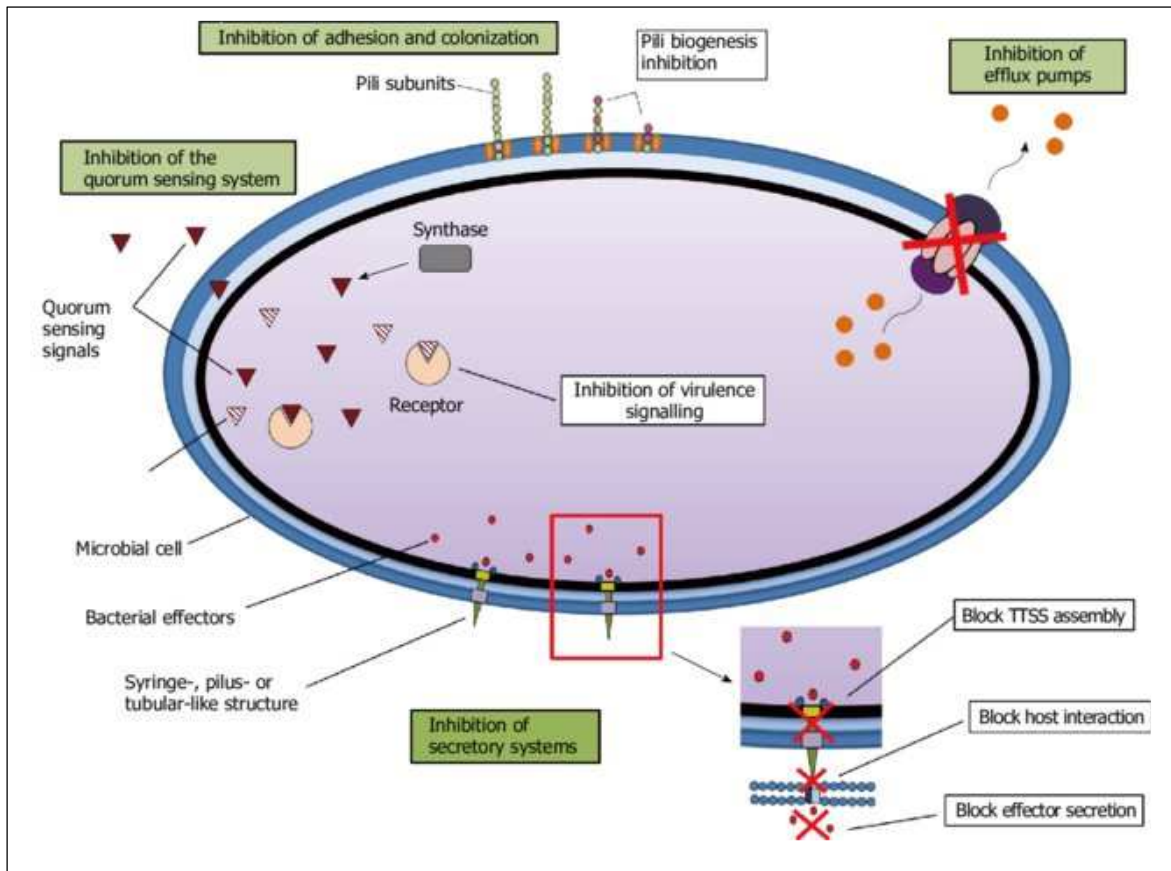


Figure 14. Representation of some anti virulence strategies targeting bacterial infections. Adapted from López *et al.*, 2014).

Therefore, agents targeting bacterial adherence by interfering pili biogenesis, flagella or adhesin formation (i.e bi-cyclic 2 pyridones), or the use of monoclonal antibodies that target the binding epitope would not only deny access of bacteria into host tissues, but also facilitate rapid clearance of pathogen and prevent the release of toxins.

3.2. Interference with bacterial communication systems

The interference with cell-cell communication between bacteria species, technically known as “quorum quenching” (QQ) or “quorum sensing inhibition” (QSI) has attracted much attention as a new approach to curtail bacterial pathogenicity and ameliorate resistance spread (**Figure 15**). Quorum sensing is a three-tier process where the bacteria synthesizes auto-inducer signal molecules which are released from the cells and sensed by cytoplasmic or membrane bound receptors at a certain threshold of bacterial density (Ng and Bassler, 2009). This in turn induces the elaboration of virulence factors including biofilm formation and antimicrobial resistance. By regulating genes that are involved in persistence and virulence, quorum sensing poses as a crucial target for anti-virulence therapy. For developing biological screening assays that will aid in the identification and isolation of inhibitors of quorum sensing, certain properties are required for an effective applicability of the compound. An ideal quorum sensing inhibitor is a low molecular weight compound of which the activity causes a decrease in expression of QS-regulated genes. Secondly, the inhibitor should exhibit a high level of specificity for the QS-regulator without toxicity effects on either bacteria or eventual eukaryotic host. In addition, the QSI should exhibit chemical stability and resist elimination by the host organism.

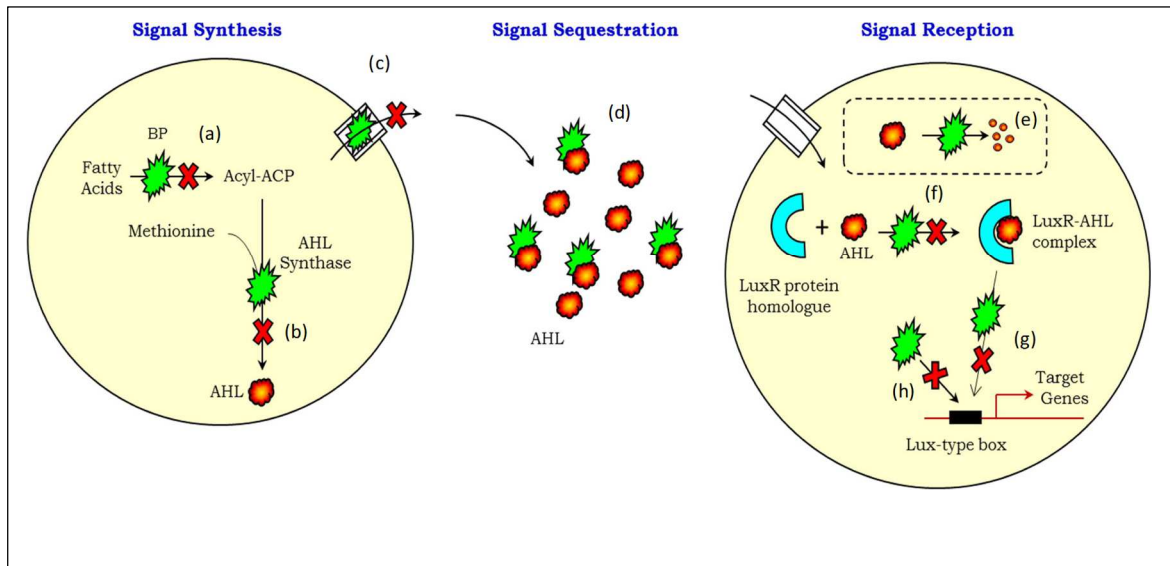


Figure 15. Possible targets and intervention strategies of disrupting bacterial quorum-sensing. Interference with QS can be achieved through the inhibition of signal synthesis, sequestration of signals or prevention of signal as represented in the diagram (a) Prevention of the synthesis of fatty acyl-acyl carrier protein (ACP), a major substrate for AHL synthase; (b) downregulation of *in vitro* AHL production following the decrease in Acyl-ACP synthesis and (c) blockage of transport proteins across cell membrane necessary for AHLs to achieve quorum. (d) sequestration of the QS-signaling molecules. In the case of signal reception, (e) AHLs can be degraded to prevent 'signal turnover', (f) Inhibition of the binding of LuxR homologue with AHLs to prevent LuxR–AHL complex formation; (g) inhibition of the activation of target gene formed by the complex; (h) direct blockage of target gene (Adapted from Singh *et al.*, 2015).

Several strategies for the identification of compounds with QS-inhibitory activities have been tested. One method that has gained more attention is the use of an AHL biosensor strain (lacking the *luxI* synthase homologue) which possess a LuxR homologue QS-controlled promoter fused to a reporter gene. When such a biosensor strain encounters exogenous AHL molecules, the activated QS-controlled *luxR* promoter induces the expression of the reporter. Therefore, in the mixture of a QSI and exogenous AHL, the transcription of the AHL-induced promoter is decreased or inhibited with consequent reduction or inhibition of the reporter signal

(Rasmussen *et al.*, 2005; Rasmussen and Givskov, 2006). A slight restriction to this screening process is that other factors apart from QSI molecules may elicit signal reduction. For instance, the challenge of the biosensor strain with sub-minimal concentrations of a toxic substance (such as metals or antibiotics), can cause growth defects, spawning a misleading effect on the reporter synthesis.

To circumvent the challenge encountered by this procedure, Rasmussen and colleagues constructed a QSI selector (QSI) based screening system (Rasmussen *et al.*, 2005). The QSI system is a biosensor strain (lacking the *luxI* synthase homologue) which possess a QS-regulated LuxR homologue promoter with a killer gene that encodes a toxic gene product (Rasmussen *et al.*, 2005). The exogenous addition of AHL activates the expression of the killer gene leading to the arrest of the bacterial growth. Conversely, the presence of the QSI-molecule rescues bacterial growth since the killer gene expression is decreased. This system was proved useful in isolating natural inhibitors of quorum sensing (including mostly enzymes, compounds, extracts of plants and fungi, antibodies from natural sources) and synthetic compounds with QSI-activities (Gokalsin *et al.*, 2017).

3.2.1. Inhibition of signal biosynthesis

The production of auto-inducer molecules can be suppressed through the inhibition of QS-signal synthases themselves or their precursors which involves, targeting the substrates of AHL synthesis (Dong *et al.*, 2007) such as the acyl-ACP (acyl-acyl carrier protein), an intermediate in fatty acid biosynthesis, through the use of a fatty acid biosynthesis inhibitor (FabI) or the inhibition of S-adenosylmethionine (SAM) using its analogues (for example, butyryl-SAM and sinefungin) (Hanzelka and Greenberg, 1996; Parsek *et al.*, 1999). In addition, several genes that prevent the accumulation of the transcription of *luxI* homologues have been identified and targeted. In *P. aeruginosa*, a *dksA* gene homologue has been isolated and found to

repress the *rhII* synthase gene (Branny *et al.*, 2001), also, the *qscR* repressor gene is well known to target the *lasI* gene and hence, modulates QS-signal synthesis and virulence factor elaboration (Chugani *et al.*, 2001). Other genes repressing signal synthesis like *rsaL* have also been reported (Rampioni *et al.*, 2006).

3.2.2. Degradation of auto-inducer molecules and inactivation

This can be achieved either by chemical, enzymatic degradation or metabolism of AHL. Chemical degradation can occur in cases where alkaline pH (above 7.0) causes the opening of the AHL lactone ring whereas acidic pH conditions lead to the recyclization (Rasmussen and Givskov, 2006). Another example of chemical degradation occurs in the reaction of oxidized AHL signal molecules like 3-oxo-C₁₂-HSL with halogen compounds such as hypochlorous and hypobromous acids. For instance, the oxidized halogen compounds produced by marine algae *Laminaria digitata* when secreted interferes with the QS-regulated gene expressions of the colonizing bacteria (Borchardt *et al.*, 2001). Enzymatic signal degradation can occur through hydrolysis of the AHL lactone ring by AHL lactonases such as AiiA, AttM, QsdA, AiiM, derived from bacterial species like *Bacillus* spp, *Agrobacterium* spp, *Klebsiella* spp among others (Chen *et al.*, 2013). Mammalian paraoxonases are regarded as lactonase-like enzymes and have been reported to inactivate the biological activity of 3-oxo-C₁₂-HSL and cause its degradation in murine tracheal epithelial cells (Stoltz *et al.*, 2008). In addition, acylases cleave the amide bond existing between the homoserine lactone and acyl side chain of AHLs. *P. aeruginosa* possess three AHL acylases (Grandclement *et al.*, 2016). The activities of amidase encoding genes *pvdQ*, *quip*, and *hacB* have been well described in literature (Huang *et al.*, 2006; Sio *et al.*, 2006; Wahjudi *et al.*, 2011). The enzyme specificity relies on the length of acyl chain and substitution on the third position of acyl chain (LaSarre and Federle, 2013). Oxidoreductases are another set of enzymes that oxidize or reduce acyl side chain of AHL and function without degradation through modification of

the C₃ keto group of the fatty acid side chain of AHL molecules. For example, BpiB09, an NADP-dependent oxidoreductase obtained from a metagenomic clone was reported to inactivate AHL-mediated biofilm, production in *P. aeruginosa* (Weiland-Brauer *et al.*, 2016). In addition to the aforementioned AHL quorum quenching enzymes, the 2-4 dioxygenase (Hod) enzyme catalyzes the conversion of PQS to *N*-octanoylanthranilic acid and carbon monoxide. The exogenous supply of Hod derived from *Arthrobacter spp* to *P. aeruginosa* PAO1 cultures leads to the inhibition of key virulence factors and tissue destruction in a leaf infection model (Pustelny *et al.*, 2009).

More so, quorum-quenching antibodies are capable of inactivating *P. aeruginosa* signals by targeting mainly AHLs. Monoclonal or polyclonal antibodies have been employed in Immuno-pharmacotherapeutic strategies to attenuate virulence and QS systems. It was reported that the immunization with an AHL protein conjugate enhanced survival of mice in a *P. aeruginosa* lung infection model (Miyairi *et al.*, 2006).

3.2.3. Prevention of signal reception and response

Competitive inhibitors such as AHL based QS- analogue molecules that prevent the binding of AHL-molecules to its LuxR-type receptor can be used (Hentzer *et al.*, 2003). These QS inhibitors exhibit significant structural biochemical diversity and can be derived from natural sources such as bacteria, algae, fungi, plants, and animals.

3.2.4. Prevention of the binding of regulator protein to DNA

The knockout of the *P. aeruginosa* *qslA* anti-activator gene was reported to increase virulence factor expression. QslA interacts with LasR in a manner that prevents the binding of LasR to its target promoter (Seet and Zhang, 2011). Therefore, QslA serves as an inhibitor of QS. Further investigations on the binding

mechanism of QlsA and LasR confirmed that the crystal structure of QslA was able to bind to LasR N-terminal ligand-binding domain (Fan *et al.*, 2013). They illustrated that QslA blocks the LasR dimerization interface, thereby disconnecting LasR from its target promoter.

3.3. Interference with regulators of global virulence

The elaboration of pathogenicity factors is an energy-consuming process and therefore requires the utilization of control circuits to avoid unnecessary energy expenditure and to optimize biological fitness. An advantage of targeting the signal transduction and regulatory mechanisms is that these components are specifically conserved to bacteria cells and not present in host eukaryotic cells. Various conserved sensory and regulatory components that act at transcriptional, post-transcriptional and/or post-translational levels have been identified in *P. aeruginosa*, which includes specific and global transcriptional controls, RNA-based regulatory mechanisms, and signal transduction and sensory mechanisms. These regulatory networks play key roles in decision making process and cover up to 10% of the genes present in the genome of this bacterium (Stover *et al.*, 2000).

Several global transcriptional regulators of virulence conserved within bacteria and specific for the control of prokaryotic gene expression have been described in *P. aeruginosa*. *P. aeruginosa* regulatory network comprises of about 690 genes and up to 1020 regulatory interconnections between their products making it the third largest reported transcriptionally regulatory network in bacteria (Galan-Vasquez *et al.*, 2011).

Among the global transcriptional regulators of virulence with interests as possible drug target is the Virulence factor regulator (Vfr). Vfr is a homologue of the

global transcriptional regulator cAMP repressor protein (Crp) which is a major regulator of genes involved in carbon source utilization in *E. coli* (West *et al.*, 1994). Vfr regulates the Las-QS system (Albus *et al.*, 1997), which in turn activates the Rhl QS system. Another strategy to attenuate *P. aeruginosa* virulence is to block c-di-GMP signaling, which plays a major role in biofilm development through the activation of the synthesis of adhesin and exopolysaccharide matrix components with consequent prevention of bacterial motility (Thompson and Malone, 2020). c-di-GMP is synthesized by diguanylate cyclases and considered as a suitable anti-virulence or antibiofilm target. For instance, ginger extracts were shown to attenuate c-di-GMP production with consequent reduction in biofilm production (Kim and Park, 2013). The GacA/GacS two-component system has been shown to modulate virulence factor production and the switch between acute and chronic *P. aeruginosa* infections (Reimann *et al.*, 1997; Chambonnier *et al.*, 2016; Francis *et al.*, 2017). Iberin, a Sulphur-containing bioactive isothiocyanate from *Armoracia rusticana* (horseradish) was shown to attenuate *P. aeruginosa* virulence factors production (rhamnolipids, chitinase, pyoverdine) by modulating the Gac/Rsm network through post-transcriptional attenuation of LadS (Tan *et al.*, 2014).

With the assistance of novel deep sequencing techniques, the complexity of transcriptional regulatory networks has been uncovered through the identification of sRNAs (small trans-acting regulatory RNAs), sensory and antisense RNAs. Majority of the RNA-based control elements influence the expression of virulence-associated processes (Oliva *et al.*, 2015), while some play redundant roles and are commonly used to fine-tune metabolic processes, virulence regulation, and/or adaptation to stress by sequestering the regulatory protein, controlling RNA degradation and/or inhibiting its translation (Feng *et al.*, 2015; Vakulskas *et al.*, 2015). Two small non coding RNAs referred to as RsmY/RsmZ, control RsmA activity in *P. aeruginosa*. Noteworthy, RsmA in its free form downregulates the biosynthesis of

gene products involved in persistent colonization of the airways of cystic fibrosis individuals such as factors promoting biofilm formation and components of the type VI secretion system (T6SS) (Goodman *et al.*, 2004; Irie *et al.*, 2010). Conversely, genes associated with acute infection, including the type III secretion system (T3SS) and type IV pili are positively upregulated by RsmA (Mulcahy *et al.*, 2006; Brencic and Lory, 2009). Hence, this regulatory process governs the switch between planktonic and biofilm growth lifestyles and coordinates the switch between acute and chronic virulence phenotypes. Ajoene, a bioactive organosulphur compound from garlic is an example of an RNA regulatory inhibitor which attenuates *P. aeruginosa* quorum sensing and virulence factors production through its repressive effect on RsmY/RsmZ small regulatory RNAs (Jakobsen *et al.*, 2017)

3.4. Interference with efflux pumps

Based on the energy source that drives antibiotic export, amino acid sequence and substrate specificity, five families of efflux pump transporters (multidrug and toxic efflux (MATE,) Major facilitator (MF), ATP binding cassette (ABC), small multi-drug resistance (SMR), and resistance nodulation division (RND) have been characterized and implicated to contribute to bacterial infection and anti-microbial resistance (Rampioni *et al.*, 2017). Efflux pumps of the RND family (resistance, nodulation, cell division) allow the pathogen to internally regulate its environment by extruding toxic compounds such as metabolites, antibiotics and QS-molecules (Pearson *et al.*, 1999). Currently, four of these RND efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM are of clinical significance and well characterized in their contribution towards *P. aeruginosa* antimicrobial resistance, since they are mostly expressed in clinical isolates (Schweizer, 2003). Over the years, several compounds also known as efflux pump inhibitors have been identified to attenuate *P. aeruginosa* efflux pump activity and thus increase intracellular antibiotic

concentration. The most popular inhibitor is phenyl-arginine-beta-naphthylamide (PA β N) which was shown by to attenuate *in vivo* virulence of *P. aeruginosa* H103 in MDCK cells and clinical strains (Hirakata *et al.*, 2009; Rampioni *et al.*, 2017). In addition, the decreased production of QS signaling molecules 3-O-C₁₂-HSL and C₄-HSL, and expression of QS-dependent virulence determinants inMDR isolates of *P. aeruginosa* from urinary and wound infections, following PA β N supplementation, was reported (El-Shaer *et al.*, 2016).

3.5 Bacteriophages

Natural occurring viruses can infect bacterial cells, causing their lysis in several cases, and are termed bacteriophages. The major advantage of using a bacteriophage is that only specific bacteria are targeted. Therefore, a bacterial specie or a clinically relevant sub-group can be targeted, while sparing the beneficial microbial flora of the host. Though numerous successes have been recorded in studies demonstrating the *in vitro* and *in vivo* phage penetration, with subsequent killing of *P. aeruginosa* biofilms, there are drawbacks with the translation of the promising results into widespread clinical use. Some of the challenges include: phage virulence and selection, sterility control, storage and their specificity for individual strains, thus requiring the use of multiple phages for the treatment of infections involving different strains of the same species (Pirnay, 2020).

4. Natural plant products targeting *P. aeruginosa* virulence

4.1. Brief origin of phytochemicals

Medicinal plants are rich in secondary metabolites and represent an important source of molecules entering the composition of pharmaceutical products (Marin

and Chrestin, 2007). This source is a natural alternative that can replace antibiotics (Harikrishnan *et al.*, 2003; Immanuel *et al.*, 2004). Thereupon, extensive research has been conducted to assess the antimicrobial effect of essential oils and extracts which have shown their ability to inhibit the growth of various pathogenic microorganisms (Tichy and Novak, 2007).

The use of plants for medicinal purposes dates to the beginning of the human species. As with humans and animals in their ecosystem, plants have been continually exposed to bacteria and have developed several mechanisms for protecting and eradicating pathogens, including via the synthesis of secondary metabolites as antimicrobials (Dixon, 2001). Although plants lack defense cells and an immune system comparable to higher animals, they are able to recognize elicitors or pathogenic signals and respond appropriately to stimuli by strengthening the cell wall, synthesizing secondary metabolites, pathogenesis-related proteins and lytic enzymes (War *et al.*, 2012). These secondary metabolites are organic compounds that can be stored in inactive, constitutive (phytoanticipins) or released in response to pathogenic attack also known as phytoalexins (Furstenberg-Hagg *et al.*, 2013). Phytoanticipins are present before a pathogen attack or produced just after the pathogen attack from pre-existing precursors (VanEtten *et al.*, 1994) phytoalexins (such as terpenoids and polyphenols) are synthesized following recognition of elicitors resulting from exposure to a pathogen attack (Hemaiswarya *et al.*, 2008). Although the use of plants in traditional medicine dates to human existence, despite everything, some of these phytochemicals have been neglected due to unfavorable or high minimum inhibitory concentrations (Ahmad *et al.*, 2015). However, increased microbial resistance associated with strong clinical demand has stimulated recent interest in phytochemicals with anti-quorum activities (Mazid *et al.*, 2011). In view of this, there has been increased research in plants and herbal products for novel therapeutic and anti-infective compounds that act as non-toxic

QS inhibitors, thereby controlling infections while minimizing emergence of bacterial resistance (Hentzer *et al.*, 2003). About 10% of terrestrial flowering plants have been used for the traditional treatment of infections, but only a fraction of 1% has been recognized and validated (Lewis and Ausubel, 2006), which is a major indication that phytochemicals may represent a vast repertoire of new therapeutic drugs (Kumar *et al.*, 2006).

*4.2. Natural plant products tackling *P. aeruginosa* virulence*

This section is presented here below as a review manuscript that aims to compile and to provide an updated literature related to the wide range of phytochemicals reported against the major virulence traits (pigments, exoenzymes, toxins, surfactants, and motility) of *P. aeruginosa*. These plant-derived bioactive compounds belong to diverse chemical classes including alkaloids, polyphenols, terpenoids, organosulphurs, phytosterols and fatty acids among others. Moreover, in this review, it is intended to describe the molecular mechanisms of action whenever data are available.

AIMS and SCOPE

In a global context of increasing antimicrobial resistance, new alternative therapies to antibiotics are needed to fight multi-resistant infections. Like animals and humans, plants are naturally exposed to bacterial infections and may respond to bacterial stimuli by producing phytochemicals or secondary metabolites (Zaynab *et al.*, 2018) for defensive purposes, which could have promising applications to combat not only phytopathogens but also human pathogens. The alternative strategies that are now being developed centers on the interference with the elements that are involved in the persistence or toxicity of *P. aeruginosa* and mainly target the virulence factors of the bacteria or the regulators that control their biosynthesis (Smith *et al.*, 2017). Unlike current antibiotics, these molecules do not directly target bacterial viability and the risk of selection pressure leading to the acquisition of resistance should therefore be greatly reduced.

This thesis is part of the main theme of the Laboratory of Microbiology Signals and Microenvironment (LMSM EA 4312, University of Rouen Normandie), namely the study of the Role of communication and environmental or eukaryotic factors in bacterial adaptation and virulence". It is included within two major thematic fields, namely "Sensor and transducer systems" led by Prof. S. CHEVALIER and "Communication in the human microbiota", led by Prof. O. LESOUHAITIER. This thesis contributed to the development of a new research axis within the LMSM, which aims to develop new strategies to fight against the opportunistic pathogen of humans *P. aeruginosa* from active ingredients of plant origin (S. CHEVALIER) or animal (O. LESOUHAITIER). In addition, it led to the establishing of new collaborations between Prof. S. CHEVALIER (LMSM, University of Rouen Normandie), Dr. Raphaël GROUGNET, Dr. Sabrina BOUTEFNOUCHET and Dr.

Sergio ORTIZ (Natural Products, Analyzes and Synthesis Team (PNAS), CiTCoM UMR 8038 CNRS, Paris, Université de Paris, Faculté de Pharmacie), and Prof. Jean Luc WOLFENDER and Dr. Luis QUIROS (Phytochemistry and Bioactive Natural Products, School of Pharmaceutical Science, EPGL, University of Geneva, Geneva, Switzerland).

Plant samples were collected, extracted and fractionated by the Pharmacognosy team of Dr R. GROUGNET; the chemical dereplication of plant extracts and fractions was carried out by Prof. WOLFENDER's team and their biological activity on *P. aeruginosa* as well as their potential mechanism of action were evaluated within the LMSM.

My thesis was funded by a joint sponsorship obtained from the French Government, Alex-Ekwueme Federal University (AEFUNAI), Nigeria and Campus France.

In this context, the objectives of my thesis are to:

- (1) To determine the attenuating effects of the virulence of *P. aeruginosa* of extracts, fractions and compounds of plants isolated from Chile or Algeria (European Union Program H2020-MSCA-RISE-2015 EXANDAS (Exploitation of aromatic plants' by-products for the development of novel cosmeceuticals and food supplements, Grant Agreement 691247).
- (2) To decipher the potential underlying mechanism of action.

The procedure used for each extract is described in the diagram shown in **Figure 16**.

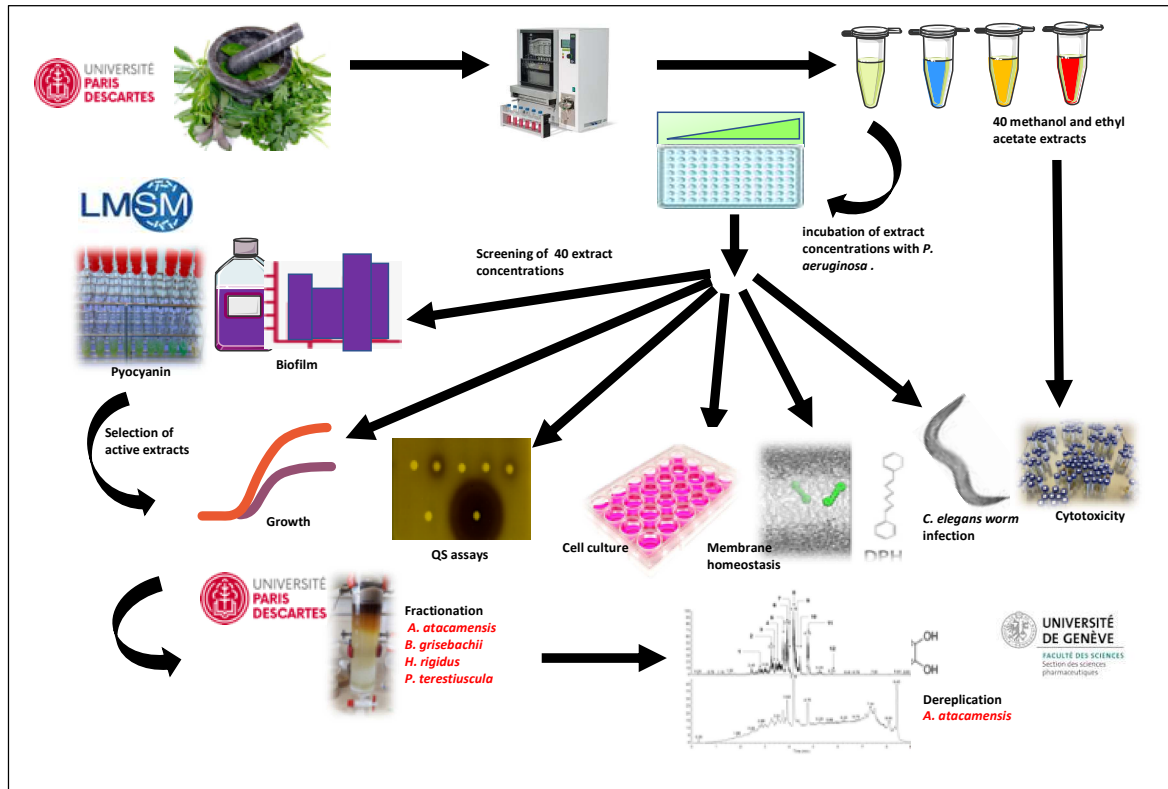


Figure 16. Overview of thesis research.

The results of this experimental work are presented in 2 parts. The first part is devoted to the study of fruit extracts of *Pistachia lenticus* from Algeria, which target the virulence of *P. aeruginosa*, work which is recorded in article 1 of this manuscript. The second part of this manuscript concerns the screening of 18 plants native to Chile for potential virulence attenuation or biofilm formation in *P. aeruginosa*. In the first article, the search for anti-virulence effects of the ethyl acetate fraction of extracts of *Azorella atacamensis* will be presented, results which are reported in a submitted paper to the journal "Biomolecules-MDPI" (*biomolecules-1014572*). In the second

part, the work concerning the attenuating effects of the virulence of *P. aeruginosa* by extracts of 3 plants originating in Chile, *Parastrephia teretiuscula* Cabrera, *Baccharis grisebachii* Hieron, and *Haplopappus rigidus* Phil. will be described. These results are presented as an article in preparation which will be supplemented by an analytical chemistry study aimed at guiding the identification of bioactive compounds. Finally, following a discussion and perspectives section, an "Annex" section will present the 3 published articles to which I contributed.

RESULTS

PART I. *Pistachia lentiscus* AS A SOURCE OF COMPOUNDS ATTENUATING *P. aeruginosa* VIRULENCE

Pistachia lentiscus

Pistacia lentiscus (Anacardiaceae), also known as the mastic tree, is a shrub that grows in the scrubland of Mediterranean climates. It is widely distributed in certain countries of North Africa (Morocco, Algeria, Tunisia, Libya, Egypt) and Mediterranean Europe (Spain, Portugal, France, Italy, Croatia, Albania, Greece) (Zohary, 1952; Al-Saghir and Porter, 2012). It is an evergreen plant of the Anacardiaceae family, which bears red fruits at the early stage and then turns black (**Figure 17**). Medicinal applications of Mediterranean varieties of *P. lentiscus* L. have been reported for centuries. In Greece *Pistacia lentiscus* var. *chios* grows in the Greek island of Chios and is mainly used for its gum called mastic (resin), for dentistry and gastrointestinal problems. In North Africa, more particularly in Algeria, the oil from the fruits and seeds is used for the treatment of respiratory tract infections and as ointments for joint pain, burns and ulcers (Bozorgi *et al.*, 2013; Rauf *et al.*, 2017). Despite the use of this plant in these areas in traditional medicine, the medicinal properties of *Pistacia lentiscus* remain under-explored.



Figure 17. Picture showing *Pistachia lentiscus* fruits (adapted from Landau *et al.*, 2014).

The objective of this study was to investigate a potential anti-virulence effect of fruit extracts of *P. lentiscus* L., against *P. aeruginosa* and its potential molecular mechanism of action. The harvest of the fruits as well as the extraction of the fruits of *P. lentiscus* L. was carried out by our collaborators at the "Natural Products, Analyzes and Syntheses" team (PNAS, CiTCoM UMR 8038 CNRS, Paris, University of Paris, Faculty of Pharmacy), between September and October 2016 and 2018 in the northern region of Algeria (Wilaya Jijel) as part of the EXANDAS project (European Union Program H2020-MSCA-RISE-2015 EXANDAS (Exploitation of aromatic plants' by-products for the development of novel cosmeceuticals and food supplements, Grant Agreement 691247). Fruits were extracted using 4 solvents (cyclohexane, ethyl acetate, methanol and water) which generated 4 extracts denoted PLFE1-PLFE4 This study reports that the cyclohexane extract of the fruit of *P. lentiscus* L. (PLFE1) attenuates the virulence of *P. aeruginosa* by mainly targeting the

production of pyocyanin by interfering with the production of communication molecules of the HAQ family. The anti-virulence activity of the PLFE1 extract could be associated with an alteration of membrane homeostasis by the modulation of the extracytoplasmic sigma factor SigX, involved in the stress response of the envelope in *P. aeruginosa*. A thorough chemical analysis of the PLFE1 extract coupled with a biological analysis has identified ginkgolic acid (C17: 1) and hydroginkgolic acid (C15: 0) as the main bioactive compounds that can interfere with the membranes of *P. aeruginosa*.

When I arrived at the laboratory, this work had been initiated by Dr. A. Tahrioui. I contributed to this work by carrying out the cytotoxicity and pyocyanin tests of the extracts and fractions, as well as the survival tests of *Caenorhabditis elegans* following the treatments of *P. aeruginosa* with the extracts. All this work has been documented in the article published in *Frontiers in Microbiology* (<https://doi.org/10.3389/fmicb.2020.01068>).

PART II. *P. aeruginosa* VIRULENCE ATTENUATION BY TRADITIONAL MEDICINAL PLANTS FROM CHILE

1. Collection of medicinal plants from Taira Community (Atacama, Chile)

Due to the extreme aridity of the Atacama region, the inhabitant plant species stand out for their exceptional ability to adapt to this extreme environment (Morong, 1891). Therefore, these plants can possess unique phytochemicals with anti-pathogenic effects. The collection of plant material was carried out by Dr Sergio Ortiz during his doctorate, under the supervision of Dr S. Boutefnouchet and R. Grounet. This collection was carried out in agreement with the representatives of the Taira community on two occasions, from September 11 to 13, 2015 and from September 9 to 11, 2016. Eighteen plants were collected and deposited in the herbarium of CONC from University of Concepcion (Concepción, Chile) and of the Pharmacognosy team of the University of Paris, Faculty of Pharmaceutical and Biological Sciences. **Table 1** shows the list of these 18 plant species collected in the Taira community, the family of plants to which they belong, their use in traditional medicine as well as their code name according to the type of solvent used for the extraction.

Table 1. List of plants used in this study and their use in traditional medicine in the Taira community (Chile) (adapted from Ortiz *et al.*, 2019)

Name	Family	Medicinal use	Solvent	code
<i>Lampayo medicinalis</i> Phil.	<i>Verbanaceae</i>	Cold, cough, stomach ache, rheumatism, bladder, kidneys, prostate, dysentery, ovarian pain, diarrhea	AcOEt	SE1
			MeOH	SE2
<i>Baccharis tola</i> Phil. ssp <i>tolu</i>	<i>Asteraceae</i>	Cold, cough, stomach pain, rheumatism, dysentery	AcOEt	SE3
			MeOH	SE4
<i>Azorella atacamensis</i>	<i>Apiaceae</i>	Cold, cough, pain, relaxation, diabetes	AcOEt	SE5
			MeOH	SE6
<i>Polylepis tarapacana</i> Phil.	<i>Rosaceae</i>	Cough, urinary tract infection, kidney, altitude sickness, heart	AcOEt	SE7
			MeOH	SE8
<i>Junellia seriphioides</i>	<i>Verbanaceae</i>	Cold, cough, fever, urinary tract infection, stomach pain, pain, bone pain, bladder	AcOEt	SE9
			MeOH	SE10
<i>Fabiana squamata</i> Phil.	<i>Solanaceae</i>	Stomach pain, anti-inflammatory, bone pain, dysentery	AcOEt	SE11
			MeOH	SE12
<i>Fabiana denudata</i> Miers	<i>Solanaceae</i>	Common cold, cough, stomach pain, anti-inflammatory drugs, bone pain, dysentery	AcOEt	SE13
			MeOH	SE14
<i>Krameria lappacea</i>	<i>Krameriaceae</i>	Common cold, urinary tract infection, bladder, kidney, gonorrhea	AcOEt	SE15
			MeOH	SE16
<i>Baccharis calliprinos</i> Griseb.	<i>Asteraceae</i>	Anti-inflammatory, bone pain	AcOEt	SE17
			MeOH	SE18
<i>Baccharis grisebachii</i> Hieron.	<i>Asteraceae</i>	Stomach pain, bone disorders taken as tea or infusions	AcOEt	SE19
			MeOH	SE20
<i>Haplopappus rigidus</i> Phil.	<i>Asteraceae</i>	Taken as infusion in treatment of cold, cough, menstrual pain, diuretics, renal troubles, heartaches, gastritis, digestive disorders and as aphrodisiac	AcOEt	SE21
			MeOH	SE22

Results

<i>Chuquiraga atacamensis</i> Kuntze	<i>Asteraceae</i>	Common cold, cough, fever, urinary tract infection, abortion, menstrual cycle control, stomach pain, pain, bladder, ovarian pain	AcOEt	SE25
			MeOH	SE26
<i>Senecio nutans</i> Sch. Bip	<i>Asteraceae</i>	Cold, cough, fever, urinary tract infection, stomach pain, pain, altitude sickness.	AcOEt	SE27
			MeOH	SE28
<i>Parastrephia teretiuscula</i> Cabrera	<i>Asteraceae</i>	Stomach pain, bone disorders taken as tea or infusions	AcOEt	SE29
			MeOH	SE30
<i>Cortaderia speciosa</i> Stapf	<i>Poaceae</i>	Cold, cough, fever, pain, ovarian pain	AcOEt	SE33
			MeOH	SE34
<i>Aloysia deserticola</i>	<i>Verbanaceae</i>	Cold, cough, fever, urinary tract infection, stomach pain, pain, kidney, antidepressant, heart, vomiting, diarrhea, milk production	AcOEt (leaves)	SE35
			MeOH (leaves)	SE36
			AcOEt (stem)	SE37
			MeOH (stem)	SE38
<i>Artemisa copa</i> Phil. Var. <i>copa</i>	<i>Asteraceae</i>	Stomach pain, pain, bone pain, diarrhea	AcOEt	SE39
			MeOH	SE40
			AcOEt	SE41
<i>Ephedra breana</i> Phil.	<i>Ephedraceae</i>	Common cold, stomach pain, rheumatoid arthritis, bladder, kidney, prostate	MeOH	SE42
			MeOH	SE43
			Basic	SE44

2. Screening of plant extracts biological activities against *P. aeruginosa*

The plant material was subjected to solvent extraction under pressure, and supercritical fluid extraction was performed as needed by the pharmacognosy team (UMR 8038, University of Paris). Pressurized solvent extraction has been used because of its high extraction efficiency: this approach requires minimal time and uses little solvent. Using methanol and ethyl acetate as a solvent, 40 extracts (arbitrarily labelled SE1-SE44) from 18 plants belonging to 8 families (**Table 1**) were evaluated for their effects of attenuating the virulence of *P. aeruginosa*. These extracts were solubilized in DMSO (1% final) and tested at a concentration range of between 0.8 and 100 µg / mL. The *P. aeruginosa* H103 strain, which is a prototrophic strain of the PAO1 strain, was used in this study to assess their effect on *P. aeruginosa* in terms of pyocyanin production on the one hand and biofilm formation on the other.

2.1. Screening for pyocyanin production attenuation

Pyocyanin is a major virulence factor in *P. aeruginosa* and is considered as a marker of QS activity (Dietrich *et al.*, 2006). To evaluate the effect of the plant extracts on the production of pyocyanin, the H103 strain was incubated in LB medium at 37 °C in the absence (1% of DMSO) and in the presence of extracts (solubilized in 1% final concentration of DMSO), with continuous agitation at 180 rpm for 24 hours in a microplate. Bacterial growth was determined by reading the absorbance at 580 nm. The quantification of pyocyanin was carried out by adding to the bacterial culture supernatant, a volume of chloroform, before adding ½ volume of 0.5 M HCl to the organic fraction. The absorbance of the acid fraction (red-pink layer) was measured at 520 nm and was related to bacterial growth (Essar *et al.*, 1990; Tahrioui *et al.*, 2020). The results are presented in **Table 2** in the form of a "heatmap" and represent the percentage of pyocyanin production compared to the control strain. Thus, the untreated control (H103 cultured in LB containing 1% DMSO) corresponds to the value of 100%.

Table 2. Effect of plant extracts obtained from Taira Atacama's community on pyocyanin production by *P. aeruginosa* H103.

Scientific name	Plant part	Plant Family	Solvent of extraction	Code	Concentrations ($\mu\text{g/mL}$) ^a							
					100	50	25	12.5	6.25	3.125	1.625	0.8
<i>Lampaya medicinalis</i> Phil.	leaves and stems	Verbanaceae	AcOEt	SE1	46	48	59.5	71	75.5	83.1	79	88.6
			MeOH	SE2	78.2	77	81.3	93	85.7	90.2	93.9	89.2
Baccharis tola Phil. ssp <i>tola</i>	Leaves and stems	Asteraceae	AcOEt	SE3	44	56	66	89	77.2	79	78	99
			MeOH	SE4	70.1	78	81.4	91	93.9	101	101	116
<i>Azorella atacamensis</i>	Leaves and stems	Apiaceae	AcOEt	SE5	28.2	43	65.2	79	88	94.7	106	95.6
			MeOH	SE6	105	98	99	93	92.6	98.2	96.2	100
<i>Polylepis tarapacana</i> Phil.	Leaves and stems	Rosaceae	AcOEt	SE7	127	105	99.6	96	92.2	89.6	90.9	86
			MeOH	SE8	44.8	53	66.2	73	72.1	82.6	77.1	89.5
<i>Junellia seriphioides</i>	Leaves and stems	Verbanaceae	AcOEt	SE9	90.5	93	92.9	105	102	100	101	112
			MeOH	SE10	56.7	63	59.9	79	62.6	62.5	70.7	78
<i>Fabiana squamata</i> Phil.	Leaves and stems	Solanaceae	AcOEt	SE11	55.8	57	71.6	81	86.9	77.6	80.4	95.3
			MeOH	SE12	47.5	60	58.5	70	72.9	91.6	80.5	91.6
<i>Fabiana denudata</i> Miers	Leaves and stems	Solanaceae	AcOEt	SE13	58.3	72	76	94	88.2	102	101	106
			MeOH	SE14	119	118	122	124	117	119	117	112
<i>Krameria lappacea</i>	Roots	Krameriaceae	AcOEt	SE15	119	111	112	98	117.4	97.4	99.6	97.4
			MeOH	SE16	146	146	154	141	125.6	110	102	102
Baccharis calliprinos Griseb.	Leaves and stems	Asteraceae	AcOEt	SE17	45.2	61	64.5	71	77.2	91.6	90.2	79.8
			MeOH	SE18	105	104	106	107	107.2	107	103	106

Results

<i>Baccharis grisebachii</i> Hieron	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE19	45.2	63	68.5	80	81.58	79.8	77.7	88.5
			MeOH	SE20	74.8	84	88.5	87	96.76	86.4	84.4	87.2
<i>Haplopappus rigidus</i> Phil.	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE21	37.6	49	55.6	74	78.5	87.1	92.3	97.9
			MeOH	SE22	38.9	51	63.3	90	78.3	89.2	86.9	94.8
<i>Chuquiraga atacamensis</i> Kuntze	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE25	64.3	69	74.9	71	75.7	85.3	74.5	102
			MeOH	SE26	74.7	78	74	72	78.9	85.6	91.2	114
<i>Senecio nutans</i> Sch. Bip	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE27	38.1	55	66	67	73.9	77.2	84.2	85.1
			MeOH	SE28	51.9	57	62.4	73	71.9	70.5	76.4	76.9
<i>Parastrephia teretiuscula</i> Cabrera	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE29	39.9	41	66	76	78.7	91.8	99	90.5
			MeOH	SE30	102	90	89	94	90.7	95.8	91	102
<i>Cortaderia speciosa</i> Stapf	Leaves and stems	<i>Poaceae</i>	AcOEt	SE33	64.6	67	71.5	76	67.8	83.3	83.2	80.9
			MeOH	SE34	77	69	82.6	81	89.5	88	83.3	85.4
<i>Aloysia deserticola</i>	Leaves	<i>Verbanaceae</i>	AcOEt	SE35	102	102	93.9	84	91.4	103	116	121
			MeOH	SE36	79	89	80.4	88	89	84.7	90.1	100
	Stem		AcOEt	SE37	51.6	62	65.7	77	76.8	84.4	88	94
			MeOH	SE38	84.2	71	83.2	70	78	75.7	83.6	88.2
<i>Artemisa copa</i> Phil. Var. <i>copa</i>	Leaves	<i>Asteraceae</i>	AcOEt	SE39	40.5	53	67.9	73	74	83	82.7	100
			MeOH	SE40	67.7	67	74.2	77	71.5	66.9	78.9	77.9
	Stem		AcOEt	SE41	73.3	79	72.9	91	82.22	91.4	92.4	75.4
			MeOH	SE42	120	97	92.9	94	97.55	97.4	96.3	102

Results

<i>Ephedra</i>	Stem	<i>Ephedraceae</i>	MeOH	SE43	195	179	160	128	107.2	95.9	112	102
<i>breana</i> Phil.			Basic	SE44	227	177	159	134	121.7	125	122	108

^a The pyocyanin production is highlighted in red to green corresponding to the lowest pyocyanin production to the highest one, respectively. The relative pyocyanin concentration quantified in response to *P. aeruginosa* treatment (expressed as a percentage compared to the untreated *P. aeruginosa*), is indicated in each case in response to 100 µg/mL of plant extracts. For example, *Baccharis grisebachii* Hieron. Ethyl acetate extract (SE19) treatment led to production of 45.18 % of pyocyanin, indicating that this plant extract led to attenuate pyocyanin production by about 54,8%. In bold are represented the 8 plants belonging to the *Asteraceae* family. In grey and bold are highlighted the 12 extracts with pyocyanin production attenuation of more than 50%.

The best attenuation results therefore correspond to red boxes in this table, and range from 28% for the ethyl acetate extract of *Azorella atacamensis* (i.e. an attenuation of pyocyanin production of 72%) to 227% for the crude extract of *Ephedra breana* Phil (an overproduction of pyocyanin of 127%). Extracts that reduce pyocyanin production by 50% or more are considered potential attenuators.

In general, the extracts exhibited various effects on the production of pyocyanin H103. Of these 40 extracts, twelve reduced the production of pyocyanin from *P. aeruginosa* H103 by 50% or more (**Table 2**). These are the ethyl acetate extracts of *Lampaya medicinalis* Phil. (SE1, 54% reduction), *Baccharis tola* Phil. ssp *tolia* (SE3, 55%), *Baccharis calliprinos* Griseb. (SE17, 55%), *Baccharis grisebachii* Hieron. (SE19, 55%), *Haplopappus rigidus* Phil. (SE21, 62%), *Senecio nutans* Sch. Bip (SE27, 62%), *Parastrephia teretiuscula* Cabrera (SE29, 60%), *Artemisa copa* Phil. Var. *copa* (SE39, 59% for the leaves) and *Azorella atacamensis* (SE5, 72%), and methanol extracts of *Haplopappus rigidus* Phil. (SE22, 61%), *Fabiana squamata* Phil. (SE12, 52%) and *Polylepis tarapacana* Phil. (SE8, 55%). The ethyl acetate extracts therefore appeared to exhibit greater anti-pyocyanin activity compared to their methanol counterparts. In general, this effect was dose-dependent, the best effect being observed for a concentration of 100 µg/mL of crude extract. Remarkably, of these 12 active extracts, 8 correspond to plant extracts belonging to the *Asteraceae* family, 1 to the *Verbanaceae* family, 1 to the *Apiaceae* family, 1 to the *Solanaceae* family and 1 to the *Rosaceae* family. These data suggest that in plants of the *Asteraceae* family there may be one or more compounds of interest in terms of virulence attenuation in *P. aeruginosa*.

2.2. Screening on biofilm formation attenuation

To evaluate the effect of the extracts on biofilm formation by *P. aeruginosa*, the H103 strain was cultured in static condition at 37°C for 24 hours in LB medium containing DMSO (1% final, control condition) or LB containing the extract at different concentrations (between 0.8 and 100µg/mL) solubilized in DMSO (1%

final). The biofilm formed was stained with crystal violet, before being removed with a 70% ethanol solution and measured at 405 nm by spectrophotometry. The results are expressed as a percentage of production relative to the control condition which was set at 100% and shown in **Table 3**. In this Table, the extracts which attenuate biofilm formation are shown in red, while those which exacerbate biofilm formation are shown in green. Initially, extracts considered to have an attenuating effect on biofilm formation, were those reducing this capacity by at least 50%. However, as shown in **Table 3**, no extract fell into this category according to this criterion. However, some extracts showed an interesting activity in terms of reduction of biofilm formation, but only at certain concentrations. This is in particular the case for extract SE19 (reduction between 38 and 44% between 3.125 and 12.5 µg / ml); SE21 and SE22 (reduction of between 22 and 29% or between 24 and 36%, respectively at the same concentrations as SE19); SE34 (reduction between 26 and 34% between 3.125 and 25µg / ml). Remarkably, many extracts increased the ability of *P. aeruginosa* to form a biofilm, which was, in most cases, dose-dependent. This is the case, for example, with the extract SE1, for which there is approximately 3.5 times more biofilm formed in the presence of the extract compared to the control condition.

Among the 12 extracts potentially interesting in terms of attenuation of pyocyanin production, i.e. SE1, SE3, SE5, SE8, SE12, SE17, SE19, SE21, SE22, SE27, SE29 and SE39, the 5 extracts SE1, SE8, SE12, SE27 and SE29 favored the formation of biofilm under our conditions, while the 7 extracts SE3, SE5, SE17, SE19, SE21, SE22, SE39 were relatively ineffective (<1, (times) on biofilm formation.

Table 3. Effect of plant extracts obtained from Taira Atacama's community on *P. aeruginosa* H103 biofilm formation.

Scientific name	Plant part	Plant Family	Solvent of Extraction	Code	Concentrations (µg/mL)							
					100	50	25	12.5	6.25	3.125	1.625	0.8
<i>Lampaya medicinalis</i> Phil.	leaves and stems	Verbanaceae	AcOEt	SE1 [†]	354	255	225	158	150	136	151	105
			MeOH	SE2 [†]	134	112	109	94	99	99	97	99
<i>Baccharis tola</i> Phil. ssp tola	leaves and stems	Asteraceae	AcOEt	SE3*	157	137	135	115	99	91	97	107
			MeOH	SE4*	234	209	181	149	131	118	96	86
<i>Azorella atacamensis</i>	leaves and stems	Apiaceae	AcOEt	SE5*	92	87	88	94	99	101	101	90
			MeOH	SE6 [†]	180	159	133	149	133	108	117	127
<i>Polylepis tarapacana</i> Phil.	leaves and stems	Rosaceae	AcOEt	SE7 [†]	252	188	174	119	97	101	80	98
			MeOH	SE8 [†]	254	184	132	101	130	116	119	97
<i>Junellia seriphioides</i>	Leaves and stems	Verbanaceae	AcOEt	SE9 [†]	298	307	253	207	125	114	100	111
			MeOH	SE10 [†]	205	151	121	132	119	96	97	121
<i>Fabiana squamata</i> Phil.	Leaves and stems	Solanaceae	AcOEt	SE11 [†]	275	222	168	153	124	110	95	96
			MeOH	SE12 [†]	284	185	123	88	116	105	103	88
<i>Fabiana denudata</i> Miers	Leaves and stems	Solanaceae	AcOEt	SE13*	184	129	117	106	111	110	115	109
			MeOH	SE14*	107	103	114	109	111	111	110	104
<i>Krameria lappacea</i>	Roots	Krameriaceae	AcOEt	SE15 [†]	199	176	145	125	110	110	114	89
			MeOH	SE16*	151	91	94	88	90	95	97	85
		Asteraceae	AcOEt	SE17*	177	133	122	110	105	109	94	111

Results

<i>Baccharis calliprinos</i> Griseb.	Leaves and stems		MeOH	SE18#	102	89	89	78	69	81	90	84
<i>Baccharis grisebachii</i> Hieron.	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE19*	119	92	70	62	56	59	74	90
			MeOH	SE20*	89	84	92	83	95	90	96	97
<i>Haplopappus rigidus</i> Phil.	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE21*	122	95	92	78	71	73	77	80
			MeOH	SE22*	133	103	78	76	70	64	75	74
<i>Chuquiraga atacamensis</i> Kuntze	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE25*	149	111	108	89	80	86	88	94
			MeOH	SE26*	144	111	92	92	96	92	99	100
<i>Senecio nutans</i> Sch. Bip	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE27*	201	134	107	108	103	101	90	98
			MeOH	SE28*	111	106	106	107	103	100	97	82
<i>Parastrephia teretiuscula</i> Cabrera	Leaves and stems	<i>Asteraceae</i>	AcOEt	SE29*	248	197	120	106	96	105	100	105
			MeOH	SE30 [†]	148	84	89	114	94	74	76	88
<i>Cortaderia speciosa</i> Stapf	Leaves and stems	<i>Poaceae</i>	AcOEt	SE33*	179	143	119	103	94	87	95	111
			MeOH	SE34 [†]	121	99	74	67	73	66	90	91
<i>Aloysia deserticola</i>	Leaves	<i>Verbanaceae</i>	AcOEt	SE35 [†]	122	219	204	135	115	110	97	112
			MeOH	SE36 [†]	186	170	126	98	85	92	96	97
	Stem		AcOEt	SE37 [†]	296	245	207	161	126	105	104	107
			MeOH	SE38 [†]	169	159	159	138	109	112	126	112

Results												
Phil. Var. <i>copa</i>	Leaves	<i>Asteraceae</i>	AcOEt	SE39*	154	123	104	124	120	112	105	96
			MeOH	SE40 [‡]	152	147	146	111	110	96	100	100
	Stem		AcOEt	SE41 [‡]	200	152	118	124	134	112	101	92
			MeOH	SE42 [‡]	97	97	106	114	106	105	104	110
<i>Ephedra breana</i> Phil.	Stem	<i>Ephedraceae</i>	MeOH	SE43 [‡]	232	146	130	108	117	110	126	126
			Basic	SE44 [‡]	147	130	116	122	103	93	112	128

[‡] Indicates assay was performed once.

Indicates assay was performed in duplicate.

* Indicates assay was performed in triplicate or more.

Although several extracts were potentially interesting, our choice fell on a more in-depth study of extracts from *Azorella atacamensis* (SE5), *Baccharis grisebachii* Hieron (SE19), *Haplopappus rigidus* Phil (SE21) and *Parastrephia teretiuscula* Cabrera (SE29). This choice was made based on different criteria: the effect on pyocyanin, the effect on biofilm formation, the amount of plant material available, and the time required to perform all the tests. Thus, among these 4 selected extracts, 2 (SE19, SE21) exhibited both an activity of attenuating the production of pyocyanin and to a lesser extent of the biofilm (at certain concentrations), 1 (SE5) reduced the production of pyocyanin without affecting biofilm formation even at a concentration of 100µg/mL, and 1 reduced pyocyanin but increased biofilm formation by a factor of approximately 2.5 times (SE29). In the latter case, the reduction in pyocyanin being of the order of 60%, we considered that different compounds could be involved in these 2 effects.

We thus continued our study on *A. atacamensis* (SE5), subject of Chapter III of this manuscript, then on 3 members of the *Asteraceae* family (Chapter IV), namely *Parastrephia teretiuscula* Cabrera (SE29), *Baccharis grisebachii* Hieron (SE19), and *Haplopappus rigidus* Phil (SE21).

3. *Azorella atacamensis* as a source of *P. aeruginosa* bio-active compounds

Azorella species are common in South America, Southeast Australia, New Zealand, and the islands of the Southern Ocean and belong to the *Apiaceae* family. The species of this family are shrubs forming a yellow-green carpet of about 15 cm. Among the most common species is *Azorella compacta* Phil. also known as "yareta" in the Quechua language, and *Azorella atacamensis* GM Plunkett & AN Nicolas (syn. *Mulinum crasiifolium* Phil.) commonly known as Chuquicandia or Chuquican by the

Taira community located in the Atacama desert (Chile, **Figure 18**). The flowers, seeds, and resins of these plants are generally taken in the form of infusions in the treatment of urinary, digestive, bronchial and respiratory disorders (Ortiz *et al.*, 2019). The main chemical compounds isolated from various species include diterpenoids, monoterpenes, mulinenic and mulinolic acids which may exert anti-plasmodial, anti-inflammatory and antibacterial activities (Borquez *et al.*, 2014). For example, essential oils of *Azorella trifurcata* exert antibacterial activity against *Pseudomonas* and *Staphylococcus* coagulase negative species at MICs of 500 µg / ml and 1000 µg / ml respectively (Lopez *et al.*, 2018). The antibacterial effect of ethyl acetate extract from *Azorella atacamensis* against *Bacillus cereus* and *Corynebacterium minutissimum* at concentrations of 60 µg / ml and 40 µg / ml was also reported in a previous study (Ortiz *et al.*, 2019).



Figure 18. Picture of *Mulinum crasiifolium* Phil. (syn. *A. atacamensis* G.M. Plunkett & A.N. Nicolas). Adapted from Fernández *et al.*, 2017.

When screening 40 plant extracts obtained from 18 medicinal plants (Taira Atacama Community) for their attenuating effects on biofilm and pyocyanin production, the ethyl acetate extract of *Azorella atacamensis* (SE5), presented the strongest effect in terms of reduction of pyocyanin, without affecting the formation of biofilm (**Table 3**). The work presented in this chapter aimed i) to specify the anti-virulence effect of the extracts of ethyl acetate from *Azorella atacamensis* (denoted AaE) against the *P. aeruginosa* H103 strain; ii) to identify active fractions, iii) to identify bioactive compounds, and iv) decipher a potential underlying molecular mechanism.

We have shown further that the extract of *A. atacamensis* (AaE) conferred significant protection against *P. aeruginosa* in a cellular model (human lung cells A549) and in a whole organism, *Caenorhabditis elegans*. The production of several virulence factors and QS molecules decreased on exposure to this extract without affecting the growth of *P. aeruginosa*. The phytochemical profiling of the AaE extract and its fractions, obtained by combining chemical dereplication and in silico molecular lattice construction approaches, allowed the potential identification of 14 metabolites carrying a mulinan-like backbone. Remarkably, this group of diterpenoids seems to be responsible for the attenuation of the virulence of *P. aeruginosa*, probably by disruption of bacterial membrane homeostasis. The significant increase in the membrane stiffness of *P. aeruginosa*, observed following treatment with these extracts, fractions or compounds, also seems to be modulated by SigX, a sigma factor of extracytoplasmic function involved in membrane homeostasis as well as the virulence of *P. aeruginosa* (Flechard *et al.*, 2018; Chevalier *et al.*, 2019).

This work was carried out in collaboration with:

- The Natural Products, Analyzes and Syntheses Team (PNAS), CiTCoM UMR 8038 CNRS, Paris, University of Paris, Faculty of Pharmacy: collection, extraction, fractionation and purification of compounds,

- The Phytochemistry and Bioactive Natural Products laboratory, School of Pharmaceutical Science, EPGL, University of Geneva, Switzerland: dereplication and construction of molecular networks.

They were submitted for publication on November 10, 2020 in the journal "Biomolecules", in a special issue entitled: "Pharmacology of Medicinal Plants".

4. *P. aeruginosa* virulence attenuation by extracts of *Parastrephia teretiuscula* Cabrera, *Baccharis grisebachii* Hieron and *Haplopappus rigidus* Phil.

Parastrephia teretiuscula Cabrera is a small evergreen shrub 5 to 20 cm tall (**Figure 19a**) that is found widely distributed in Peru and the Andes, at high altitudes (3000 to 4200 m). The aerial parts of various species are traditionally used to treat toothaches, bone fractures and bruises. Different compounds (isofraxidin, flavonoids, esters of E-caffeoyl or E-cinnamoyl, scopoletin, p-coumaroyl-oxytremetone, umbelliferone, phenylpropanoid and phenolic compounds) have been described to exert antimicrobial effects (Dalmeida *et al.*, 2012).

Baccharis species are shrubs 30 cm to 2 m in height (**Figure 19b**), growing mainly in the Altiplano, northwestern Argentina and northern Chile. The aerial parts of these species are used in traditional medicine in infusions or in the form of a poultice for the treatment of digestive disorders, burns, bone problems or wounds in the Atacama community and in Argentina (Indena *et al.*, 1996).

The *Haplopappus* species, also known as Bailahuén, are shrubs (**Figure 19c**) that are mainly found in the Andes of Chile. The leaves and stems are commonly used in traditional medicine for the treatment of liver, gastrointestinal and choleric diseases (Schrickel and Bittner, 2001). The secondary metabolites isolated from the resinous exudates of *H. rigidus* are mainly monoterpenes, terpenoids, flavonoids or even coumarin, for example. These compounds display an antioxidant effect (Torres *et al.*, 2004; Torres *et al.*, 2006).

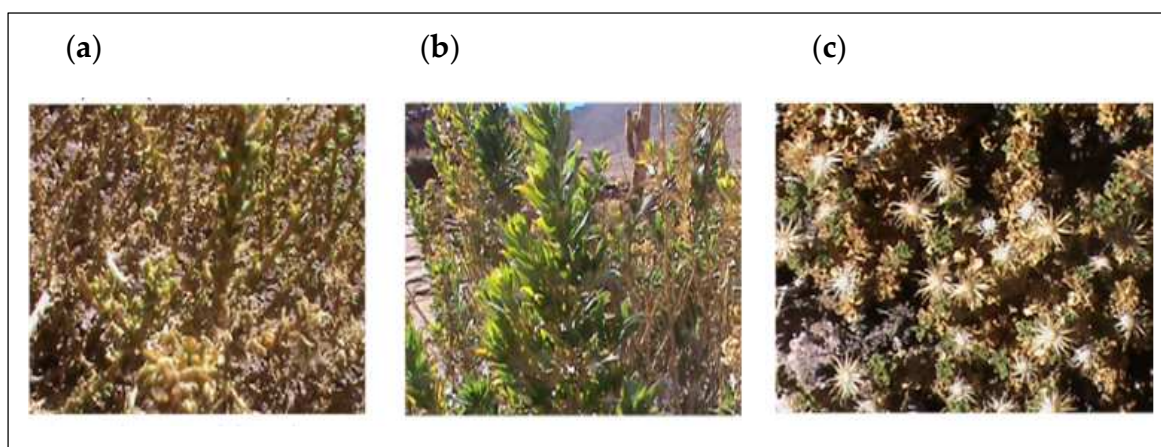


Figure 19. Pictures of *Parastrephia teretiuscula* (Kuntze) Cabrera, *Baccharis grisebachii* Hieron and *Haplopappus rigidus* Phil. Adapted from (Ortiz *et al.*, 2019a).

Screening of the 40 medicinal plants extracts revealed that:

- the ethyl acetate extract of *Parastrephia teretiuscula* Cabrera SE29 induced a 60% reduction in the pyocyanin present in a culture supernatant of *P. aeruginosa* H103 at a concentration of 100 $\mu\text{g}/\text{mL}$. Although this extract increased biofilm formation by approximately 2.5-fold at this same concentration (**Table 3**), we selected it for further study because of its significant effect on pyocyanin production. In addition, we hypothesized that different compounds could be involved in these 2 phenotypes, potentially allowing them to be considered for separation for future application.

- the ethyl acetate extract of *Baccharis grisebachii* Hieron SE19 induced a reduction of 55% of the pyocyanin present in a culture supernatant of *P. aeruginosa* H103, without affecting the formation of biofilm at a concentration of 100 $\mu\text{g}/\text{mL}$. However, at a lower concentration, between 1.625 and 25 $\mu\text{g} / \text{mL}$, this extract made it possible to reduce the capacity of *P. aeruginosa* H103 to form a biofilm, while retaining a slight effect of reducing the production of pyocyanin.

- the ethyl acetate extract of *H. rigidus* SE21 induced a 62% reduction in the pyocyanin present in a culture supernatant of *P. aeruginosa* H103, without affecting

the formation of biofilm at a concentration of 100µg/mL. However, at a lower concentration, between 0.8 and 12.5µg/mL, this extract made it possible to reduce the capacity of *P. aeruginosa* H103 to form a biofilm (between 2 and 29%), while retaining some limiting effect on the reduction of pyocyanin production.

We have therefore selected these 3 extracts for a more in-depth study of the potential virulence attenuating effect and the potential mechanisms involved. The work presented in this chapter aimed:

- i) To specify the anti-virulence effect of ethyl acetate extracts of *Parastrephia teretiuscula* Cabrera (noted PtE), *Baccharis grisebachii* Hieron (noted BgE) and *Haplopappus rigidus* (noted HrE), against the strain *P. aeruginosa* H103,
- ii) To identify active fractions,
- iii) And to decipher a potential underlying molecular mechanism.

We have shown that the 3 extracts led to a decrease of *P. aeruginosa* cytotoxicity against human lung cells without affecting the growth of *P. aeruginosa* nor showing toxicity to lung cells. Likewise, a decrease in the production of the virulence factors pyocyanin and elastase was observed after exposure to plant extracts. The HAQ-like quorum sensing system (PQS) is affected by plant extracts and stiffening of the bacterial membrane has been observed after exposure to the extracts. Active fractions have been identified. The extracts and fractions are currently being dereplicated, to guide the type of compounds to be purified. These results will make it possible to complete the manuscript in preparation presented below.

This work was carried out in collaboration with:

- The Natural Products, Analyzes and Synthesis Team (PNAS), CiTCoM UMR 8038 CNRS, Paris, University of Paris, Faculty of Pharmacy: collection, extraction, fractionation

- The Phytochemistry and Bioactive Natural Products laboratory, School of Pharmaceutical Science, EPGL, University of Geneva, Switzerland: de-replication and construction of molecular networks under analysis

DISCUSSION / FUTURE DIRECTIONS

1. The urgency of novel strategies for controlling antibiotic resistance

Today, clinicians face a significant limitation of options for treating infections caused by *Pseudomonas aeruginosa*. The search for new molecules to fight against this bacterium is therefore of crucial importance, as indicated by the WHO in 2017 (Global Priority List of Antibiotic-Resistant Bacteria, World Health Organization, 2017). *P. aeruginosa* was recently listed by WHO as one of the pathogens that cause the greatest infectious disease threats facing physicians today. Indeed, the absence of therapeutic tools in the face of the emergence of strains resistant to certain antibiotics or even Pan-Drug-Resistant (resistant to all antibiotics) has become a reality and the search for new antibacterial agents is therefore a challenge in case of public health, as demonstrated by the creation of the priority program to fight against antibiotic resistance decided by the French government at the end of 2018, a program coordinated by INSERM on behalf of the entire national scientific community.

Virulent pathogens such as *P. aeruginosa* are able to colonize and survive in the human body or medical devices (contact lenses, mechanical heart valves, intrauterine devices, pacemakers, prosthetic joints, urinary catheters among others) through its ability to express multiple virulence factors. For over a decade, antibiotics, although inefficient against biofilm forming infections, have been utilized as weapon against these pathogenic infections, however, the alarming resistance of pathogens to conventional antibiotics represents one of the greatest challenge to the success of modern medicine. Current studies attribute 700,000 deaths annually to drug resistant pathogens and estimates ~10 million deaths by the

year 2050, if urgent actions are not taken (O'Neill, 2016). Assuming that the victory against bacterial infection depends on its ability to optimally express virulence properties, anti-virulence or anti-infective strategies, which disarm the bacteria of its pathogenic factors may likely provide promising alternative solutions for controlling the emergence and spread of drug-resistant pathogens. Alternative strategies such as the use of phages, bacteriocins, antimicrobial peptides among others, are considered by researchers in the fight against pathogenic infections (Ahmad *et al.*, 2017; Oliveira *et al.*, 2020; Pirnay, 2020). Interestingly, novel approaches are proposed that do not alter growth or viability of the pathogen but focus on disarming the production of virulence factors, causing biofilm disruption, neutralizing bacterial toxins or disrupting secretion systems, and more prominently, quenching of bacterial QS system (Rasko and Sperandio, 2010; Grandclement *et al.*, 2016; Muhlen and Dersch, 2016). The advantages of this anti-virulence mechanism over conventional antibiotics lies in the fact that anti-virulence compounds target only essential pathways involved in host–pathogen interaction rather than viability, hence, limiting the selective pressure for development of resistant mutants (Rasko and Sperandio, 2010; Grandclement *et al.*, 2016; Muhlen and Dersch, 2016). Moreover, anti-virulent effect is specific against the bacteria causing pathogenesis and should preserve the constituent normal flora, whilst giving the immune system ample time to eradicate invading bacteria. In addition, anti-virulence therapy enlarges the repertoire of pharmacological targets that can generate antimicrobials with novel mechanisms of action and can improve the lifespan of already existing antibiotics.

2. Plants as source of metabolites with anti-virulence compounds

Like animals and humans in the ecosystem, plants are continuously exposed to pathogenic attack and environmental stresses. Contrary to animals and humans with elaborate humoral and cell-mediated defence system against pathogenic infection, plant defences rely on the secretion of phytochemicals or secondary metabolites (including terpenoids, flavonoids, glycosteroids) (Zaynab *et al.*, 2018). These secondary metabolites are small molecules of low molecular weight (<500) derived from primary metabolic pathways. But unlike the primary metabolites, these are not necessary for plant survival, but they play major roles in reproduction, interspecies competition and defense, including the protection of plants against attack from herbivores and pathogens (War *et al.*, 2012; Furstenberg-Hagg *et al.*, 2013; Zaynab *et al.*, 2018) Plant products create countless opportunities for novel drug leads (e.g efflux pump inhibitors, anti-biofilm agents, anti-cancer) owing to the production of wide range of secondary metabolites with chemical diversities. It is estimated that over five hundred thousand plant species are in existence (Borris, 1996), despite this huge number, only, 5-15% of higher plants have been studied for the presence of bio-active components, which is an indication that nature's biodiversity is highly underexplored (Cragg *et al.*, 1997). Plant species have played major role in disease prevention and treatment since the origin of human existence, of which majority of modern medicines are derivatives of traditional medicinal plants. The presence of various phytochemicals such as alkaloids, lectins, flavonoids, phenolics, terpenoids, polypeptides, tannins and polyphenols (Perumal Samy and Gopalakrishnakone, 2010) may be advantageous for anti-infective drug development. The benefits of the application of plant-derived therapy is the perception that compared to chemically synthesized drugs, they are relatively safer with long history of traditional use in infection treatment. In addition, plant derived medicines offer wider mechanism of action and more economical treatment (Sandasi

et al., 2010). The aim of utilising plant products as sources of therapeutic agents are: to identify and isolate its active constituents for direct application as drugs e.g. reserpine (a plant-derived indole-alkaloid), to obtain active molecules from novel structures and applying them as lead compounds for the production of new patentable entities with enhanced activity and/ or minimal toxicity, to use part of plant or its whole product as a pharmacological tool (e.g. mescaline) or herbal remedy e.g Ginkgo (*Ginkgo biloba*) and St. John's wort (*Hypericum perforatum*). Their mode of action against microbes rests on their chemical and structural properties and affects microorganisms in diverse ways which includes; the interference with synthesis of RNA/DNA, interaction with membrane proteins, leakage of cellular constituents through the destabilization of proton motive force, interference with bacterial communication system, efflux and biofilm inhibitory activity etc.

3. *Pistachia lentiscus* L., *Azorella atacamensis*, *Baccharis grisebachii* Hieron, *Haploppapus rigidus* Phil and *Parastrephia teretiuscula* Cabrera as metabolites sources with virulence attenuating activity

Different plant extracts belonging to various families of plants were explored in this study for their anti-virulence effects against the human pathogen *P. aeruginosa*. These extracts were first screened for their antibiofilm and anti-pyocyanin activities. While most of the extracts did not show a great attenuation effect on biofilm formation, *Pistachia lentiscus* L., *Azorella atacamensis*, *Baccharis grisebachii* Hieron, *Haploppapus rigidus* Phil and *Parastrephia teretiuscula* were selected for their effect on pyocyanin production reduction. Indeed, we show herein that the ethyl acetate extracts of these plants exhibited 82%, 72%, 55% 62% and 60% pyocyanin production attenuation by *P. aeruginosa*, respectively. Noticeably, the attenuation of pyocyanin activity by the extracts in our study are in agreement with previous studies for other plants. For example, the ethyl acetate fraction of *Acacia hockii* belonging to *Fabaceae*,

used in Bénin traditional medicine decreased pyocyanin production by 45.55%, when used at sub-MIC of 100 μ g/mL (Tchatchedre *et al.*, 2019). Similarly, the ethanolic extract of *Plantago asiatica* L, commonly used in traditional Chinese Medicine displayed a dose dependent inhibitory pyocyanin activity at sub-MICs from 8-16 μ g/mL on *P. aeruginosa* PAO1 (Li *et al.*, 2019). Also, (Husain *et al.*, 2018), demonstrated significant reduction of *P. aeruginosa* PAF79 pyocyanin by methanol extracts of *Psorelea corylifolia*.

In addition to the pyocyanin and biofilm formation, elastase and rhamnolipids are major virulent factors during lung infection. Elastase is controlled by the *lasI-lasR* and *rhll-rhlR* network (Brint and Ohman, 1995; Hentzer and Givskov, 2003), whereas rhamnolipid biosynthesis is under *rhll-rhlR* control, driven by an operon comprising of RhlA and RhlB gene products. In this study, the attenuation of elastolytic activity of *P. aeruginosa* by *A. atacamensis*, *B. grisebachii*, *H. rigidus* and *P. teretiuscula* Cabrera ethyl acetate extracts, are similar with previous findings on *Ocimum sanctum*, *Manilkara zapota* and *Musa paradisiaca* extracts (Musthafa *et al.*, 2010), and inhibition of elastase activity by the sesquiterpene lactone dehydroleucodine at 0.12mg/mL, derived from *Artemesis douglasiana* in *Asteracea* family, (Mustafi *et al.*, 2015). Moreover, hordenine from sprouting barley was also reported to exhibit effect against the production of elastase, proteases and rhamnolipids in *P. aeruginosa* PAO1 at 0.5-1.0mg/mL (Zhou *et al.*, 2018). Following the observed inhibition of *P. aeruginosa* virulence activities by the plant extracts derived from Atacama community, it is possible to suggest the correlatation of the bioactivities of these plants with their traditional usage in treatment of gastrointestinal, urogenital and respiratory disorders. Altogether, these data showed that these extracts displayed a significant potential as *P. aeruginosa* virulence attenuators

The concept of anti-virulence therapy lies in the ability of the inhibitory agent to block the virulence regulatory pathways of the pathogen without interference with its growth or viability, in order to minimize selective pressures that can lead to resistance development (Rasko and Sperandio, 2010). However, the eventual toxicity of the inhibitory agent to host cells is of great concern in order to proceed into clinical application. Hence, the selected extracts were assessed for their effect on bacterial growth or viability and toxicity towards A549 monolayer cells. Interestingly, the selected extracts exhibited no anti-bacterial activity when tested at several concentrations. Viability of *P. aeruginosa* H103 exposed to *Pistachia lentiscus* L. fruits were not affected when assessed in flow cytometry measurement. Nonetheless, additional investigations need to be undertaken to assess whether *P. aeruginosa* can develop resistance towards these plants extracts upon repeated exposure.

In terms of toxicity towards A549 monolayer cells exposed to the extract concentrations, *B. grisebachii* extract exhibited no toxicity at all concentrations tested. However, *H. rigidus* and *A. atacamensis* extracts exhibited slight toxicity from 6hrs and 24hrs post-incubation respectively, reaching a maximal toxicity of 28% and 22% at 100µg/mL. Similarly, *P. teretiuscula* Cabrera exhibited minimal toxicity at 50µg/mL, reaching maximum toxicity of 20% at 100µg/mL after 24hrs of incubation whereas the cyclohexane extract of *P. lentiscus* L. fruits exhibited moderate cytotoxicity from 12.5µg/ml, after 3hrs post-incubation. Altogether, our data show that the assayed extracts indicated slight or no cytotoxicity against A549 human monolayer cells and without any effect on *P. aeruginosa* H103 bacterial growth.

The *in vivo* virulence attenuation of these selected extracts was assayed in biological models, i.e. on A549 human pulmonary cells and on the nematode *C. elegans* worms in a fast kill assay, respectively. In both infection models, the virulence of *P. aeruginosa* upon exposure to plant extracts at a dose of 100µg/mL is

shown to be significantly attenuated at least partly because of decreased production of the pyocyanin virulence factor. Pyocyanin has been reported to have many damaging and toxic effects on mammalian cells most likely through a mechanism involving oxidative stress (Allen *et al.*, 2005; Hall *et al.*, 2016). However, because A549 cells lungs damage and fast killing of *C. elegans* are multifactorial, we cannot exclude that essential mediators other than pyocyanin such as hydrogen cyanide (HCN) and effectors of the type III secretion system might be involved (Mahajan-Miklos *et al.*, 1999; Gallagher and Manoil, 2001; Engel and Balachandran, 2009; Cezairliyan *et al.*, 2013). It would thus be of interest to assay production of other virulence factors such as HCN, elastase, rhamnolipids and effectors of the T3SS.

Since the production of and secretion of these virulence factors are under the control of QS regulatory system, in principle, interference with these regulatory networks will most-likely affect the elaboration of these virulence factors (Defoirdt, 2018). Three quorum-sensing networks (Las, Rhl and PQS) have been reported for *P. aeruginosa*. Las and Rhl system utilise N-acyl homoserine lactones as signalling molecules whereas the PQS system uses 2-heptyl-3-hydroxy-4-quinolone (PQS) and its precursor 2-heptyl-4-quinolone (HHQ) as signalling molecules (Diggle *et al.*, 2007; Lin *et al.*, 2018). To investigate the effect of plant extracts on HAQ and AHL production, reporter strains were used (McClellan *et al.*, 1997; Fletcher *et al.*, 2007). The biosensor strains, are unable to synthesize AHQs (Fletcher *et al.*, 2007) and AHLs (McClellan *et al.*, 1997), except through exogeneous supply, subsequently, they were assayed for bioluminescence production (HAQ QS) or violacein production (AHL QS) respectively, using crude alkyl-quinolone (AQ) and acyl-homoserine lactone extracts from *P. aeruginosa* H103 culture, grown in the presence or absence of the plant extracts. Our results show that treatment with the plant extracts caused a decrease in AHLs and/ or alkyl quinolone-QS-molecules production. These results imply that the *P. aeruginosa* H103 strain produced lower levels of QS molecules in

the presence of the extracts, suggesting that the extracts may contain bioactive compounds that interfere with the QS regulatory system. Several studies have indicated the ability of phytochemicals to attenuate bacterial QS-machinery and thereby regulate the expression of their virulence determinants. Majority of the antagonists identified from natural sources mimic AHL signals, and consequently, affect the binding of the signaling molecules (Teplitski *et al.*, 2000; Defoirdt, 2018).

The results obtained following our investigation of effect of *A. atacamensis* extract on AHL QS-molecule production, agree with other studies like (Alva *et al.*, 2019), who reported the anti-QS activities of the ethanolic extracts of some indigenous spices and vegetables *Cinnamomum verum*, *Garcinia indica*, *Luffa acutangular*, *Solanum melongena*, *Syzygium aromaticum* and *Curcuma longa* derived from Mangaluru, India. However, slight difference exists between the approach used in this study and theirs. We have detected anti-QS activity of *A. atacamensis* using exogenous crude AHLs derived from *P. aeruginosa* H103 in untreated and extract treated conditions for violacein pigment quantification in CVO26 biosensor strain. Therefore, we categorically show, the reduction in *P. aeruginosa* AHLs QS-molecules production, whereas, in the latter study, though they have reported the anti-QS activity of several extracts against *P. aeruginosa*, but their results show violacein pigment inhibition by the plant extracts against wild type *C. violacein* MTCC2656 strain, hence, the anti-QS activity of their extracts were not assayed on the reference strain *P. aeruginosa*. Also, the studies from (Vasavi *et al.*, 2016) showed that ethanol and ethyl acetate fractions from *Centella asiatica* L. inhibited QS-controlled violacein production in addition to the attenuation of pyocyanin, elastolytic and proteolytic activities in *P. aeruginosa* PAO1. Altogether, our data suggest that the plants extracts could interfere with some of the QS autoinducers production.

In the last years there was a large amount of published investigations reporting natural products highlighting their potential in targeting bacterial virulence factors (Silva *et al.*, 2016). Several plant-derived compounds that belong to alkaloids, organosulfurs, coumarins, flavonoids, phenolic acids, phenylpropanoids, terpenoids, among other chemical classes have been found to be active against pyocyanin pigment production (Silva *et al.*, 2016). However, there is still a significant lack of knowledge on the specific underlying molecular mechanisms of action. It was suspected that the lipophilic character of these plant extracts could increase membrane permeability through its diffusion across the lipid bilayer as seen with hydrophobic drugs such as macrolides (Delcour, 2009). It would thus be of interest to assay the bacterial membrane permeability in response to these plants extracts.

Since the bacterial outer membrane is in the first line of contact between the bacteria and external environment, and since membrane homeostasis has been related to its fluidity or stiffness (Flechard *et al.*, 2018; Bouffartigues *et al.*, 2020; Tahrioui *et al.*, 2020), the latter phenotype has been investigated via fluorescence anisotropy measurements. The exposure of the ethyl acetate extracts of *B. grisebachii*, *H. rigidus* Phil and *P. teretiuscula* to *P. aeruginosa* H103 led to rigidification of the membrane by up to 20.06%, 37.6% and 23.5% respectively, whereas *A. atacamensis* ethyl acetate extract and *Pistachia lentiscus* cyclohexane extract enhanced *P. aeruginosa* membrane rigidity by 26.2% and 20%, respectively. These results imply physiological changes and adaptation of the bacterial membrane in response to the exposure of the plant extracts. Previous studies have shown that the extracytoplasmic function sigma factor (ECF σ) SigX, regulates the expression of enzymes involved in fatty acid biosynthesis and involved in the maintenance of phospholipid composition and membrane fluidity (Boechat *et al.*, 2013; Duchesne *et al.*, 2013; Flechard *et al.*, 2018; Chevalier *et al.*, 2019; Bouffartigues *et al.*, 2020; Tahrioui *et al.*, 2020). Moreover, the regulation of virulence related phenotypes including

pyocyanin biosynthesis have been associated to SigX (Duchesne *et al.*, 2013; Gicquel *et al.*, 2013; Blanka *et al.*, 2014; Chevalier *et al.*, 2019). The involvement of SigX in cell wall stress response and rigidification of *P. aeruginosa* H103 membrane was identified further in *A. atacamensis* ethyl acetate and *P. lentiscus* L. extracts by monitoring the expression of sigX using a construction of transcriptional fusion of sigX promoter region coupled to a promoter-less luxCDABE cassette in a replicative pAB133 vector (pAB-PsigX). We observed a dose dependent increase in bioluminescence production following exposure to *A. atacamensis* and *P. lentiscus* L. extracts. In addition, fluorescence anisotropy measurements of PAOSX (Δ sigX deletion) mutant (Bouffartigues *et al.*, 2012) exposed or not to *A. atacamensis* extract revealed no significant effect on fluorescence anisotropy values. These results put together, possibly suggests the presence of membrane interactive compounds in some of the selected extracts.

4. Towards the identification of the bioactive compounds

The case of Pistachia lentiscus L.

From the thirteen fractions (PLFE1- PLFE 13) obtained from *P. lentiscus* L. extract, the main compounds detected from fractions PLFE2- PLFE4 were identified as ginkgolic acid (C17:1) and hydroginkgolic acid (C15:0) using Gas chromatography-Mass Spectrometry (GC-MS) with structural characterisation by 1D and 2D NMR and Ozonolysis. To extrapolate possible mechanism of action of *P. lentiscus* L. fruit extract, membrane-interactive compounds (possibly ginkgolic acid (C17:1) and hydroginkgolic acid (C15:0)) increased membrane rigidification which may lead to the activation of a cell wall stress response in *P. aeruginosa* (**Figure 20**). Whether the bioactive compounds go directly into the membrane or act on the outer membrane surface is unknown. Perhaps the ECF sigma factor SigX may be acting

through the reduction of HAQ-QS system leading to the attenuation of pyocyanin production or the effect against pyocyanin production, thus leading to *P. aeruginosa* virulence attenuation. Whether these bioactive components go directly into the membrane or act on the surface of the outer membrane is unknown. This study, however, reports the involvement of ECF sigma factor SigX in the rigidification of the *P. aeruginosa* membrane. Finally, to complete this study, it would be interesting to investigate the molecular mechanism of action regarding the bacterial response to these ginkgolic acids by RNA seq assays.

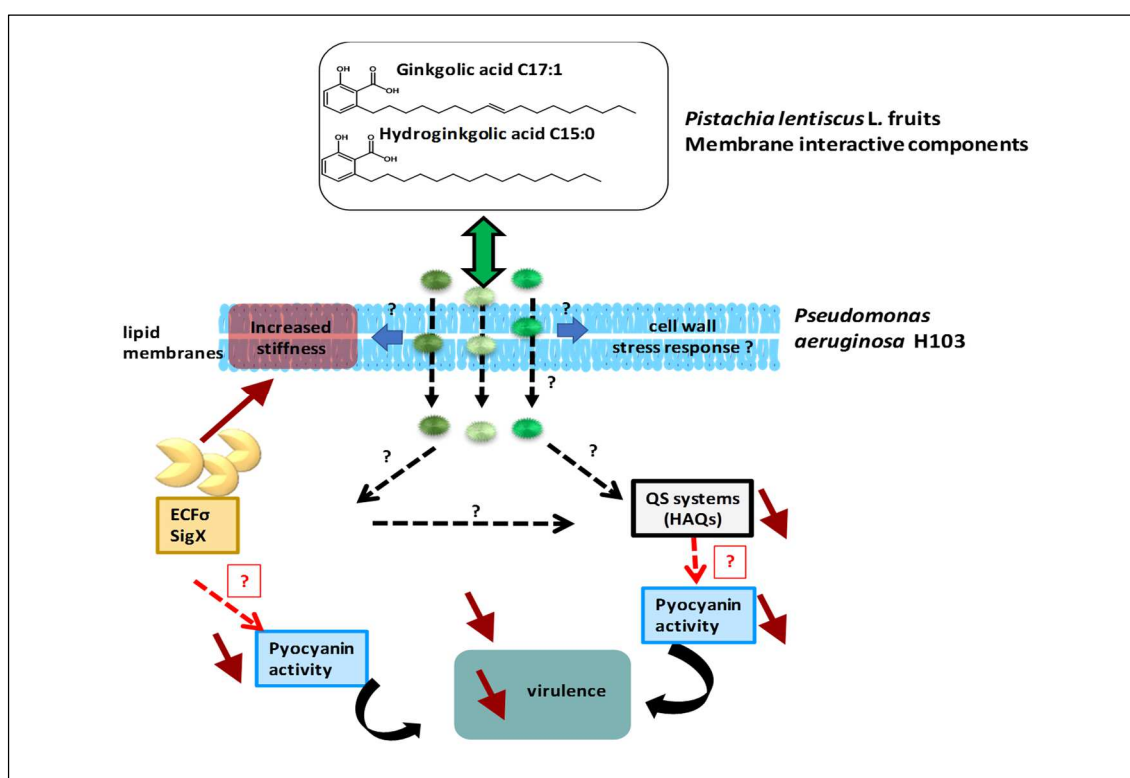


Figure 20. Putative mode of action of ginkgolic acids from *Pistachia lentiscus* on *P. aeruginosa* H103.

The case of *Azorella atacamensis*

The fourteen fractions obtained from *A. atacamensis* (AaF-1- AaF-14) and re-analysed for their anti-pyocyanin activity exhibited significant reduction in pyocyanin production except for AaF-12 and AaF-13. AaF-2 and AaF-6 exhibited the greatest anti-pyocyanin effect. Therefore, anti-virulence activities of AaF-2 and AaF-6 were further explored using AaF-12 as a control. As expected, AaF-2 and AaF-6 significantly reduced elastase and rhamnolipids virulence production and membrane fluidity while AaF-12 had no significant effect. Interestingly, the involvement of ECF sigma factor SigX, in modulation of *P. aeruginosa* H103 membrane fluidity was validated through fluorescence anisotropy measurements of *P. aeruginosa* H103 and $\Delta sigX$ mutant PAOSX exposed to untreated and treated conditions of extract or AaF-2 and AaF-6 (Figure 21).

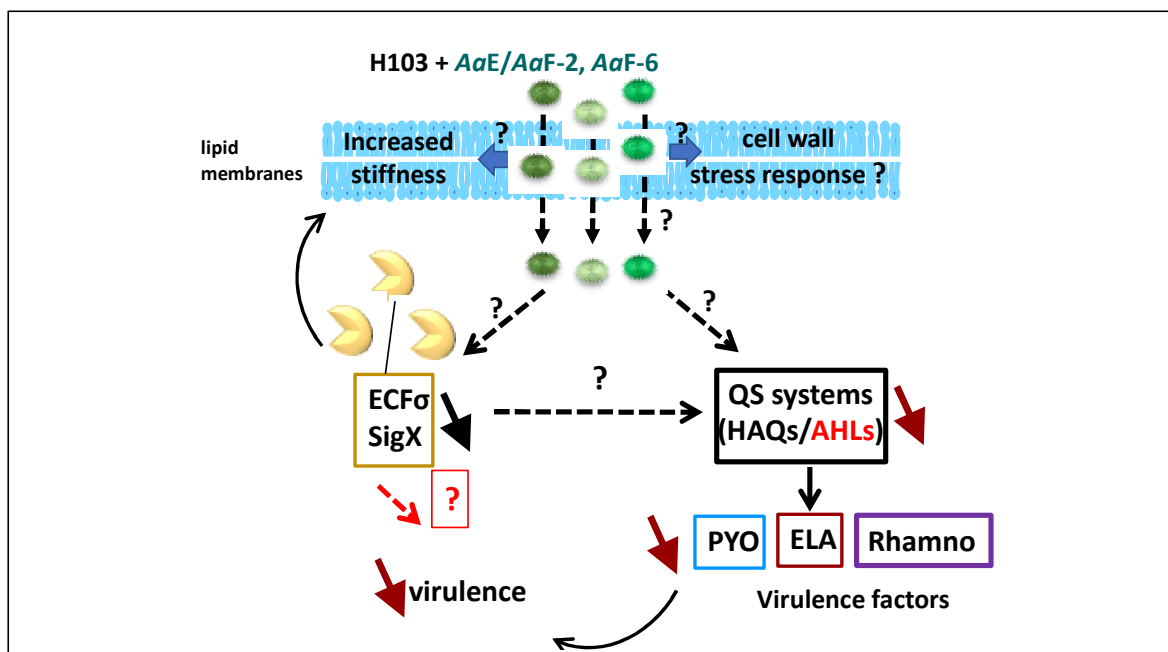


Figure 21. Putative mode of action of *A. atacamensis* extract and fractions on *P. aeruginosa* H103.

Owing to the complex composition of natural products and the draw-backs of bio-guided fractionation processes which often results to the identification of an already existing compound with described biological functions (Mohamed *et al.*, 2016), de-replication has been adopted for rapid identification of previously identified metabolites in crude natural extracts to prevent unnecessary re-isolation (Wolfender *et al.*, 2019, **Figure 22**). The presumed compounds are identified through the comparison of preliminary spectral data to the spectral database of already identified metabolites. With recent advancements in spectroscopy techniques such as in HRMS and NMR, which strengthen their sensitivity, the degree of confidence of a dereplication process has tremendously improved. In this study, dereplication of extract and fraction was performed by an UPLC-PDA-CAD-ESI-HRMS/MS in positive and negative ionization modes.

Fourteen putative compounds were identified following the de-replication of *A. atacamensis* extract and its fractions which consisted of terpenoids (mostly mulinane and azorellane type-diterpenoids, triterpenoids, sesquiterpenoids), fatty acids and lipids. Seven of these compounds have been previously isolated from *A. atacamensis* species and the other seven identified for the first time. However, the main compounds were identified as 11, 14-dioxo-12-mulinen-20-oic acid, mulinic acid, and mulinenic acid. The correlation of the phytochemical constituents with bioactivity is exemplified with the improved anti-pyocyanin activity of the less polar fractions, (*AaF*-1 to *AaF*-7) which are composed mainly of terpene-like structures, compared to the highly polar fractions (*AaF*-8 to *AaF*-14), containing mostly fatty acids, saccharolipids and prenol lipids. Putting together these results, terpene-like compounds identified in the extracts and fractions *AaF*-2 and *AaF*-6 would be responsible for the observed membrane rigidification and attenuation of *P. aeruginosa* virulence properties.

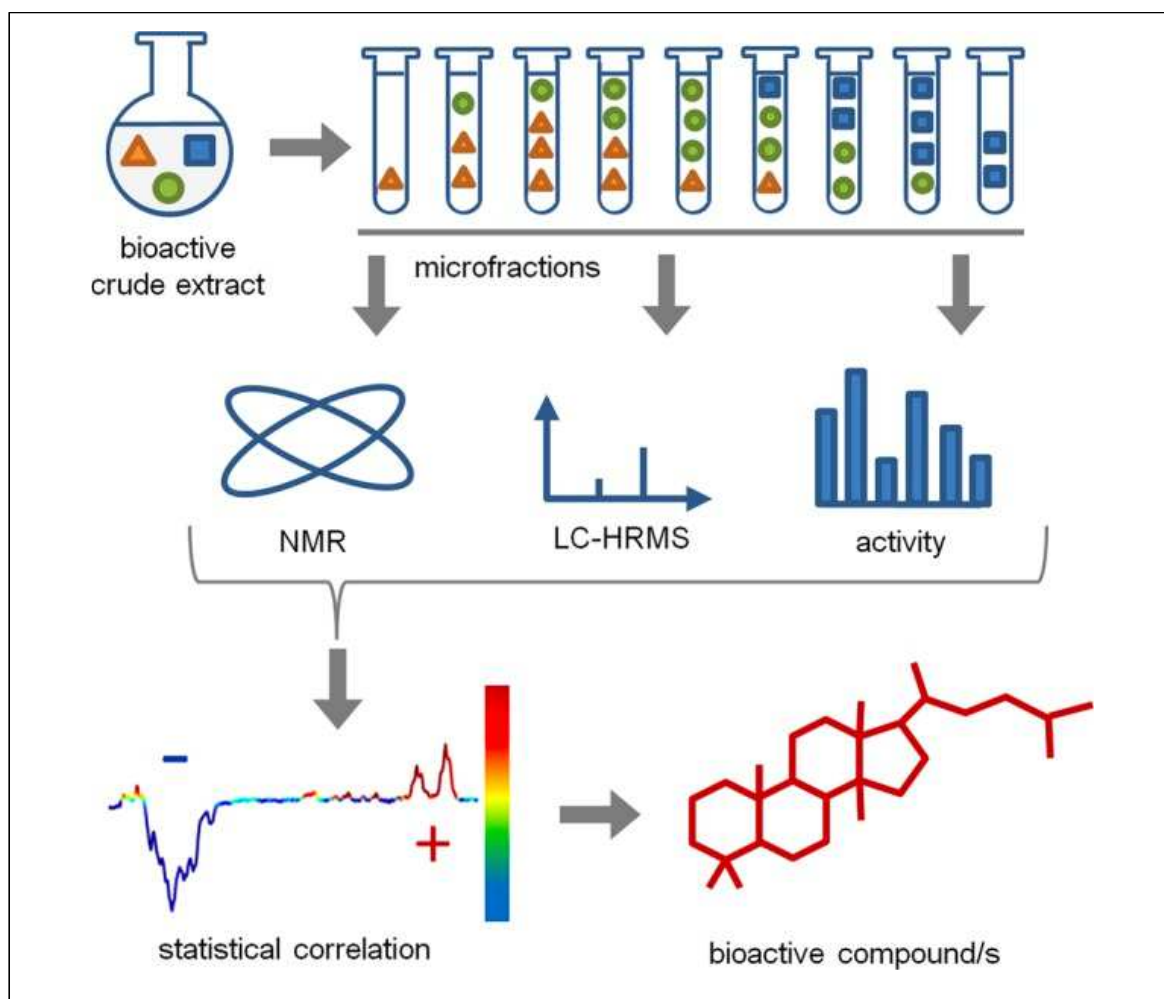


Figure 22. Pictogram showing the overview of Hetero-covariance analysis approach (adapted from Aligiannis *et al.*, 2016 and Grienke *et al.*, 2019).

Highlighting the anti-pathogenic effect of terpenoids from plant sources against *P. aeruginosa*, Oleanolic aldehyde coumarate from the tropical legume *Dalbergia trichocarpa* was reported to attenuate rhamnolipids, elastase and pyocyanin production and the expression of *las* and *rhl* QS-systems (Rasamiravaka *et al.*, 2015b). In previous studies, mulinane- and azurellane-type diterpenoids (exclusive to *Mulinum*, *Azorella* and *Laretia*) have been reported to exhibit antimicrobial, antiprotozoal and anti-inflammatory activities among others (Borquez *et al.*, 2014; Dzul-Beh *et al.*, 2020). The difference between mulinane- and azurellane-type diterpenoids lie in their skeletal arrangement. Mulinanes possess a tricyclic skeleton coupled with five, six, and seven-membered rings, with an angular substituent at every ring junction, while the azurellane skeleton harbors a tetracyclic organization. Most mulinanes are characterized by the presence of a carboxyl group at the C-20 position and a functionalized seven-member ring, whereas, in azurellane the C-13 is oxygenated while C-20 is non-functionalized. Nevertheless, this research study contributes to the limited knowledge on the target genes, target proteins, and signaling pathways associated with mulinane and azurellane diterpenoids mechanism of action and the potentials for *A. atacamensis* to be used as adjuvant in treatment of *P. aeruginosa* infections. Further identification and isolation of the bioactive compounds will now be required to investigate their precise effect and mode of action.

The case of B. grisebachii and H. rigidus, P. teretiuscula

A similar approach of dereplication is currently ongoing on *B. grisebachii*, *H. rigidus*, and *P. teretiuscula* extracts and fractions and would certainly allow to guide for identification of the possible bioactive compounds in these plant extracts.

5. Additional perspectives

This study demonstrated virulence attenuation of *P. aeruginosa* H103 strain by some plant extracts and their derived components using a rich complex media (Luria Bertani) for *P. aeruginosa* growth. Subsequent experiments should focus on testing the anti-virulence criteria in several media conditions to know if these selected extracts or their derived compounds exhibit variabilities in the QSI activity or growth activity, since the growth of pathogenic bacteria is deeply affected by media composition. In one study, the growth of *P. aeruginosa* in the presence of a QS inhibitor Furanone C-30, was shown to be decreased in a minimal media (media containing 0.1% adenosine) whereas the growth of the pathogen was unaffected in a complex rich media (Maeda *et al.*, 2012).

In addition, it will equally be necessary to ascertain the anti-virulence effect of these plant extracts on other biological models of infection such as in mouse.

Moreover, we assayed these plants extracts, fractions, and some compounds on *P. aeruginosa* H103 laboratory strain. It would be of importance to assay these phenotypes on clinical strains, including multi-drug resistant strains of *P. aeruginosa*. Also, the ability of these extract and derived compounds to potentiate antibiotic activity needs to be ascertained, since anti-virulence activity does not eliminate the infecting pathogen, and hence needs to be used as adjuvants in combination with conventional antibiotics for the treatment of pathogenic infections. Furthermore, the bioactivity of the compounds, once identified and isolated, could be modified in a drug-design strategy to improve their activity without affecting their toxicity to potentially proceed into clinical trials.

ADDITIONAL CONTRIBUTIONS

Article 1. The absence of SigX results in impaired carbon metabolism and membrane fluidity in *Pseudomonas aeruginosa*

The aim of this study was to extrapolate the role of SigX in the regulation of carbon catabolite repression. This study reveals through in-depth proteomic and transcriptomic analysis, the dysregulation of genes mostly involved in energy, transport, and carbon metabolism in a SigX mutant compared to H103 wild type when grown in LB medium. This is because of the alteration in bacterial membrane and metabolic changes which influenced the nutrient uptake in a SigX mutant. Consequently, growth of the SigX mutant in LB is altered. Based on pseudoCAP analysis (Winsor *et al.*, 2016), the genes involved in transport of membrane proteins and small molecules, fatty acid and phospholipid metabolism were shown to be downregulated while genes involved in carbon and amino acid metabolic pathways were upregulated in a *sigX* mutant (PAOSX). Additionally, the Crc (catabolite repression control) which regulates carbon uptake and utilization and previously shown to activate the expression of C4-dicarboxylate transporter (DctA), known to be associated with the uptake of two of the preferential carbon sources used by *P. aeruginosa* (succinate and fumarate), were repressed in a PAOSX mutant. These suggest the altered regulations of metabolic pathways, leading to a reduced carbon catabolite repression in the *sigX* mutant in comparison to H103 wild type. Since the *sigX* mutant is well known to modify membrane composition, the bacterial envelope was destabilized with a detergent PS80 in order to increase non-specific intake of nutrients. Interestingly, the addition of polysorbate 80 (PS80) to the LB medium restored growth defects in the PAOSX mutant including HHQ production and gene

expression levels of selected target genes of Crc/Hfq/CbrAB networks to nearly wild type levels. In addition, fluorescence anisotropy measurements showed that PS80 restored the membrane fluidity of PAOSX mutant to the same level with the wild type grown in LB or LB supplemented with PS80. Taken together, the results suggest the indirect involvement of SigX in the control of carbon catabolite repression possibly through its influence on membrane fluidity. My personal contribution to this research was the proofreading and correction of manuscript before publication at *Scientific Reports* with the ascension number: doi.org/10.1038/s41598-018-35503-3.

Article 2: Activation of the cell wall stress response in *Pseudomonas aeruginosa* infected by a Pf4 phage variant

The prophage Pf4 is integrated into PAO1 genome and has been shown to contribute to biofilm maturation and increased lysis of cells, resulting in the release of eDNA into biofilm matrix and the emergence of small colony variants. Pf4 variants are regarded as superinfective if they can re-infect and attack the prophage carrying host. This study was carried out with the aim of investigating the physiology and response of *P. aeruginosa* H103 exposed to a Pf4 filamentous phage superinfective variant through combined genotypic and phenotypic analysis. This phage variant was obtained by screening the library of *P. aeruginosa* H103 mutants by random transposon mutagenesis, which displayed a colony lysis phenotype. Following the assessment of bacterial growth and examination of the biofilm structure of H103 exposed and unexposed to the supernatant of Pf4 filamentous phage variant in static and dynamic flow conditions on a confocal laser-scanning microscope, it was observed that the Pf4 phage variant infection partially lysed H103 bacterial population, affecting its growth. Furthermore, Pf4 filamentous phage variant infection induced biofilm production by altering cellular architecture, leading to the formation of a filamentous bacterial cell morphology in the dynamic flow condition which could be as a result of a SOS response. Additionally, the infection of H103 with this Pf4 phage variant led to the abrogation of the type IV pili dependent twitching motility, the increased expression of exopolysaccharides (pel and alginates), c-di-GMP production, and increased membrane fluidity (following fluorescence anisotropy measurements), thereby triggering cell wall stress response which was shown to be mediated by the extracytoplasmic function sigma factors (ECF σ) AlgU and SigX. My personal contribution to this study was in proofreading, review and editing of manuscript before publication at multidisciplinary Digital

Publishing Institute (MDPI) journal *Microorganisms* with the ascension number:
doi.org/10.3390/microorganisms8111700.

Article 3: The temperature-regulation of *Pseudomonas aeruginosa* *cmaX-cfrX-cmpX* operon reveals an intriguing molecular network involving the sigma factors AlgU and SigX

Bacteria perceive temperature variations like heat shock or cold shock as signals, which can trigger cell wall stress response, and hence alter membrane composition. Consequently, proteins such as heat shock proteins and cold shock proteins are activated which are equally necessary in response to antimicrobial presence. The aim of this study was to show the effect of temperature variations on the expression of *cmaX-cfrX-cmpX* operon and the functional involvement of AlgU and SigX in coordinating membrane homeostasis and temperature variations. Interestingly, the study observed differential regulation of *cmaX-cfrX-cmpX* operon in *P. aeruginosa* under heat shock and cold shock conditions. This was performed by exposing H103 strain from 37°C to sudden temperature variations of 4°C and 60°C, and subsequently monitoring the gene expressions of *cmaX-cfrX-cmpX* operon in the two conditions. While mRNA levels of *cmaX-cfrX-cmpX* were increased in heat shock, only *cfrX-cmpX* were significantly upregulated in cold shock. The expression of the *cmaX-cfrX-cmpX* operon under heat shock was shown to be regulated by AlgU and activated from a *cmaX*-proximal promoter independently of RpoH, whereas in the case of cold shock, the operon is regulated from *cfrX*, from a SigX dependent promoter. The involvement of AlgU in heat shock was validated by assessing the gene expression levels of *algU* and its targets *algD*, *rpoH*, *oprF* and *amrZ*, in heat shock and cold shock conditions. While the expression levels of the genes were elevated in heat shock conditions except *amrZ*, which was reduced, in response to cold shock, there was no difference in expression level of the same genes. Moreover, a putative AlgU recognition motif similar with the AlgU consensus binding sequence was

discovered upstream of *cmaX* by visual inspection. Similarly, evaluation of gene expression of SigX target genes in a $\Delta sigX$ at 37°C and under cold shock confirmed SigX control of *cfrX-cmpX* transcriptional unit and its involvement in increased *cfrX-cmpX* transcription in response to cold shock. Furthermore, SigX was shown to be activated in the presence of a membrane modifying agent, valinomycin. Putting together, the study highlights new insights to the regulation of SigX and networks that are associated with the maintenance of envelope homeostasis.

My personal contribution to this research was the proofreading and correction of manuscript before publication at *Frontiers in Microbiology* with the ascension number: doi.org/10.3389/fmicb.2020.579495.

MATERIALS AND METHODS

I. Materials

I.1. Plant material

Eighteen plant materials were collected from Taira Atacama's community (Calama, Chile) in September 2016, in accordance with Chilean Biodiversity Strategy and Nagoya Protocol. An agreement was reached with an official transfer of plant material by the community, which was registered at INIA (Instituto de Investigaciones Agropecuarias, Ministerio de Agricultura, Chile). These plants were identified by Pr. Alicia Marticorena of the CONC Herbario, Universidad de Concepción, Chile with vouchers of each plant deposited in CONC Herbario, Universidad de Concepción, Chile and Herbarium of the Laboratory of Pharmacognosy, Paris Descartes University, France (**Table 1**).

Table 1. Plants voucher code, GPS collection site and altitude

N°	Scientific Name	Plant family	Voucher	GPS Collection Site	Altitude (m)
1	<i>Aloysia deserticola</i> (Phil.) Lu-Irving & O'Leary	Verbanaceae	MKOSB092015T1A 01	21°40'57.092" S 68°38'5.163" W	3248
2	<i>Artemisa copa</i> Phil. var. copa	Asteraceae	MKOSB092015T1B 01	21°44'35.653" S 68°38'3.476 W	3255
3	<i>Azorella Atacamensis</i> G.M. Plunkett & A.N. Nicolas	Apiaceae	MKOSB092015T2A 04	21°44'43.546" S 68°38'0.451" W	3635
4	<i>Baccharis calliprinos</i> Griseb.	Asteraceae	MKOSB092015T2B 02	21°51'30.785" S 68°38'25.015" W	3455
5	<i>Baccharis grisebachii</i> Hieron.	Asteraceae	MKOSB092015T2C 01	21°42'33.948" S 68°45'44.013" W	3242
6	<i>Baccharis tola</i> Phil. ssp tola	Asteraceae	MKOSB092015T2A 03	21°44'48.768" S 68°38'8.948" W	3244
7	<i>Chuquiraga atacamensis</i> Kuntze	Asteraceae	MKOSB092015T2D 01	21°40'38.531" S 68°40'39.885" W	4215

8	<i>Cortaderia speciosa</i> (Nees & Meyen) Stapf	Poaceae	MKOSB092015T2F 02	21°56'5.729" S 68°36'43.126"	3258
9	<i>Ephedra americana</i> Humb. & Bonpl. ex Willd.	Ephedraceae	MKOSB092015T1C 01	21°40'28.768" S 68°43'11.063" W	3265
10	<i>Fabiana denudata</i> Miers	Solanaceae	MKOSB092015T2A 09	21°44'44.407" S 68°38'55.721" W	3271
11	<i>Fabiana squamata</i> Phil.	Solanaceae	MKOSB092015T2A 08	21°43'57.193" S 68°37'38.165" W	3281
12	<i>Haplopappus rigidus</i> Phil.	Asteraceae	MKOSB092015T2C 02	21°41'53.756" S 68°46'26.963" W	3408
13	<i>Junellia seriphioides</i> (Guillem & Hook. Ex Hook.) Moldonke	Verbanaceae	MKOSB092015T2A 07	21°44'12.692" S 68°37'41.254" W	3433
14	<i>Krameria lappacea</i> (Dombey) Burdet & B. B. Simpson	Krameriaceae	MKOSB092015T2B 01	21°51'36.09" S 68°38'16.673" W	3570
15	<i>Lampaya medicinalis</i> Phil.	Verbanaceae	MKOSB092015T2A 02	21°44'48.281" S 68°38'8.948" W	3650
16	<i>Parastrephia teretiuscula</i> (Kuntze) Cabrera	Asteraceae	MKOSB092015T2E 02	21°38'12.644" S 68°50'37.863" W	3739
17	<i>Polylepis tarapacana</i> Phil.	Rosaceae	MKOSB092015T2A 05	21°44'34.074" S 68°37'55.236" W	3040
18	<i>Senecio nutans</i> Sch. Bip.	Asteraceae	MKOSB092015T2E 01	21°38'12.644" S 68°50'44.661" W	4202

I.2. Bacterial strains, plasmids and growth media

The strains used in this study along with their characteristics are listed in **Table 2**. All bacterial strains were stored in Microbank® cryotubes, a ready-to-use system designed to simplify the storage and retrieval of bacterial cultures (Pro-Lab diagnostics, Ontario, Canada).

Table 2. List of strains and plasmids used in this study

Bacteria or plasmid	Characteristics	Reference or source
<i>Pseudomonas aeruginosa</i>		
H103	Prototrophic strain of <i>P. aeruginosa</i> PAO1	Hancock et Carey, 1979
H103-pAB133	H103 harboring the plasmid pAB133, Gm ^R	This study
H103-pAB::PsigX	H103 harboring the plasmid pAB::PsigX, Gm ^R	This study
PAOSX	H103 sigX::lox	Bouffartigues <i>et al.</i> , 2012
PAO1 $\Delta pqsA$ CTX- <i>lux::pqsA</i>	<i>pqsA</i> mutant containing a copy of the <i>pqsA</i> promoter linked to the <i>luxCDABE</i> genes and inserted into a neutral site in the chromosome	Fletcher <i>et al.</i> , 2007
<i>Chromobacterium violaceum</i>		
CV026	CV017 derivative containing <i>cviI::Tn5xylE</i> , Km ^R	Mclean <i>et al.</i> , 1997
<i>Escherichia coli</i>		
TOP10	<i>F-mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\Phi 80lacZ\Delta M15 \Delta lacX74 recA1 araD139$ $\Delta(araleu)7697 galU galK rpsL (Str^R) endA1$ <i>nupG</i>	Invitrogen™
S17.1	<i>ecA pro (RP4-2Tet::Mu Kan::Tn7)</i> , donor and helper strain for conjugation	Simon <i>et al.</i> , 1983
OP50	Food for <i>Caenorhabditis elegans</i>	Stiernagle, 2006
Plasmids		
pGEM-T	High-copy-number cloning vector, Ap ^R , <i>bla</i> , <i>lacZ</i>	Promega
pAB133	pBBR1MCS-5-based cloning vector containing the promoterless <i>luxCDABE</i> operon, Gm ^R	Bazire <i>et al.</i> , 2010
pAB::PsigX	pAB133 containing the promoter region of <i>sigX</i> , transcriptional fusion, Gm ^R	This study

The culture media used in this study and their composition are described in **Table 3.**

Table 3. List of media and composition used in this study

Media	Composition	Concentration (g/L)	Source
Luria-Bertani (LB)	Yeast extracts	5	BD, Franklin Lakes, New Jersey, United States
	Bactotryptone	10	AES laboratory, Bruz, France
	NaCl	5 (LB5) 10 (LB10)	VWR, Radnor, Pennsylvania, United States
Luria-Bertani (LB) agar	Agar	20	Biomérieux, Marcy L'Etoile, France
Soft agar (1.5 %)	Agar	15	Biomérieux, Marcy L'Etoile, France
Dulbecco's Modified Eagle Medium	DMEM (base)	4.5	Lonza, Basel, Switzerland
DMEM with glutamine	Glucose+ Glutamine	0.29	Lonza, Basel, Switzerland
Complete DMEM	Fetal calf serum	10 %	Lonza, Basel, Switzerland
	Penicillin-Streptomycin	1 %	
M9 minimal medium	Na ₂ HPO ₄	6	Sigma Aldrich, Saint-Louis, Missouri, United States
	KH ₂ PO ₄	3	Alpha Aesar Haverhill, Massachusetts, United States
	NaCl	5	VWR, Radnor, Pennsylvania, United States
	MgSO ₄	0.12	Fisher Chemicals, Hampton, New Hampshire, United States
Nematode Growth medium	KH ₂ PO ₄	3.4	Alpha Aesar Haverhill, Massachusetts, United States
	MgSO ₄	0.120	Fisher Chemicals, Hampton, New Hampshire, United States
	CaCl ₂	0.111	Acros Organics, Hampton, New Hampshire, United States
	Cholesterol	0.005	Acros Organics, Hampton, New Hampshire, United States

			Hampshire, United States
	NaCl	3	VWR, Radnor, Pennsylvania, United States
	Protease peptone n°3	2.5	BD, Franklin Lakes, New Jersey, United States
	Agar	17	Biomérieux, Marcy L'Etoile, France
Peptone-Glucose-Sorbitol	Bacto-peptone	10	BD, Franklin Lakes, New Jersey, United States
	NaCl	10	VWR, Radnor, Pennsylvania, United States
	Agar	34	Biomérieux, Marcy L'Etoile, France
	Glucose	20%	
	D-sorbitol	27.4	Biomérieux, Marcy L'Etoile, France

II. METHODS

II.1. Microbiological assays

II.1.1. Growth conditions of bacterial strains

The *Pseudomonas aeruginosa* H103 (Hancock and Carey, 1979) and Δ sigX (Bouffartigues *et al.*, 2012) used are all derivatives of *P. aeruginosa* wild-type PAO1. Planktonic cultures were grown aerobically for 24 h at 37 °C in LB broth on a rotary shaker (180 r.p.m) from an initial inoculum adjusted to an O.D at 580 nm of 0.08. *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) and *P. aeruginosa* PAO1 Δ pqsA CTX-lux::pqsA (Fletcher *et al.*, 2007) were used as reporter strains for quorum sensing molecules detection. *C. violaceum* CV026 was grown at 28 °C in LB medium

with orbital shaking at 180 r.p.m. The PAO1 Δ pqsA CTX-lux::pqsA was cultured at 37 °C in LB medium supplemented with 125 μ g/mL of tetracycline on a rotary shaker (180 r.p.m). The antibiotics stock solutions used in this study were sterilized by filtration through 0.22- μ m filters, aliquoted into daily-use volumes and kept at -20 °C. Each set of experiments was performed at least three times.

II.1.2. Bacterial growth monitoring

To assess the effect of the plants extracts on *P. aeruginosa* H103 strain growth kinetics. Bacterial cultures were grown in the absence or presence of plants extracts in specialized white sided and transparent flat bottom 96-well microtitre plate (BD Falcon, San Jose, California, United States). Then, the growth of *P. aeruginosa* was monitored at 37 °C over the course of 24 h using the Spark 20M multimode Microplate Reader, equipped with an active temperature regulation system (Te-Cool™, Tecan Group Ltd., Switzerland). Absorbance at 580 nm was recorded every 15 min. The bacterial growth curve for untreated cultures (control condition) and treated cultures was determined by plotting the values against time.

II.1.3. Virulence factor quantification

II.1.3.1. Pyocyanin quantification assay

To evaluate pyocyanin production, *P. aeruginosa* H103 cells untreated (DMSO 1 %, v/v) or treated with plant extracts at various concentrations were grown in LB broth in a 96-well microtiter plate at 37 °C for 24 h with shaking (180 r.p.m). After incubation for 24 h, cell growth was determined by measuring the O.D at 580 nm.

Pyocyanin quantification assay was carried out as described previously (Essar *et al.*, 1990). Briefly, one volume of chloroform was used to extract free-cell supernatants samples. Then, ½ volume of 0.5 M HCl was added to the chloroform layer (blue layer). The absorbance of the HCl layer (red-pink layer) was recorded at 520 nm using the Spark 20M multimode microplate reader controlled by SparkControl™ software Version 2.1 (Tecan Group Ltd., Männedorf, Switzerland) and the data were normalized for bacterial cell density (A580nm).

II.1.3.2. Elastase quantification assay

The LasB elastolytic activity of *P. aeruginosa* H103 untreated or plant extracts treated cultures was assessed using previously described protocol (Pearson *et al.*, 1997) with minor modifications. In brief, 50 µL aliquot of untreated or treated *P. aeruginosa* H103 cell free-supernatant was added to 950 µL of Elastin congo red buffer (100 mM Tris-HCl, 1 mM CaCl₂ at pH 7.5) in 10 mg of Elastin Congo red substrate (Sigma Aldrich, USA). The mixture was allowed to incubate at 37 °C for 18 h on a rotary shaker (180 r.p.m). Next, the reaction was stopped by adding 100 µL of 0.12 M EDTA. Insoluble substrate was sedimented by centrifugation and supernatant absorbance was recorded at 495 nm.

II.1.3.3. Rhamnolipids quantification assay

The production of rhamnolipids was quantified following a methylene-blue based method (Rasamiravaka *et al.*, 2016). Briefly, 500 µL of cell-free supernatants from *P. aeruginosa* H103 grown in the absence or presence of plant extracts at various concentrations were acidified to 2.3 ± 0.2 pH using 1 M HCl solution. Rhamnolipids were extracted twice with equal volume of ethyl acetate. The organic layer was dried in a speed vac rotatory evaporator and residue were dissolved in 500 µL of

chloroform mixed with 50 μ L of methylene blue solution [1.4 % w/v methylene blue in 95 % ethanol adjusted to 8.6 ± 0.2 pH by addition of 15 μ L of a 50 mM borax buffer]. The organic phase was transferred to a new tube and mixed with $\frac{1}{2}$ volume of 0.2 M HCl solution before absorbance quantification at 638 nm.

II.1.4. Static biofilm formation assay

To assess the propensity of *P. aeruginosa* H103 strain to form biofilms in the presence of plant extracts at various concentrations, a crystal-violet-adhesion assays was performed as described by O'Toole (2011). Briefly, overnight cultures were inoculated into a fresh LB broth and grown at 37 °C for 24 h in a 96-well microtiter plate under static conditions. Cell growth was determined at 580 nm. The biofilm was measured by discarding the medium, rinsing the wells with water and staining any bound cells with 0.1 % crystal violet. The dye was dissolved in 30 % (w/v) acetic acid and optical density was determined at 595 nm using the Spark 20 M multimode microplate reader controlled by SparkControl™ software Version 2.1 (Tecan Group Ltd., Männedorf, Switzerland). In each experiment, the background staining was adjusted by subtracting the crystal violet bound to uninoculated controls.

II.1.5. Virulence attenuation assay using A549 pulmonary cell infection model

The human lung A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, BioWhittaker®) supplemented with 4.5 g/L glucose, L-Glutamine, 10 % heat-inactivated (30 min, 56 °C) fetal bovine serum (FBS), and 100 Units mL⁻¹ each of penicillin and streptomycin antibiotics. Cells were grown at 37 °C under the atmosphere of 5 % CO₂ and 95 % air with regularly medium change

until a confluent monolayer was obtained. The anti-virulence effect of plant extracts on *P. aeruginosa* H103 was determined using an enzymatic assay (Pierce™ LDH Cytotoxicity Assay Kit, Thermo Scientific™), which measures lactate dehydrogenase (LDH) released from the cytosol of damaged A549 cells into the supernatant. After overnight incubation with *P. aeruginosa* H103 (10^8 CFU/mL) previously treated or untreated (control condition), the supernatants from confluent A549 monolayers grown on 24-well tissue culture plates were collected and the concentration of the LDH release was quantified. A549 cells exposed to 1X Lysis Buffer were used as a positive control of maximal LDH release (100% lysis) as specified by the manufacturer's recommendations. The background level (0% LDH release) was determined with serum free culture medium.

II.1.6. Virulence attenuation assay using *Caenorhabditis elegans* infection model

The *C. elegans* wild-type Bristol strain N2 worms were grown at 22°C on nematode growth medium (NGM) agar plates using *Escherichia coli* OP50 as a food source. The *P. aeruginosa*-*C. elegans* fast-kill infection assay was performed as described previously (Blier *et al.*, 2011) with minor modifications. Briefly, cultures of *P. aeruginosa* H103 strain untreated or treated with plant extracts were seeded on a 24-well plate containing in each well 1 mL of Peptone-Glucose-Sorbitol (PGS) agar. Control wells were seeded with 25 µL of *E. coli* OP50 from an overnight culture. The plate was then incubated at 37°C for 24 h to make bacterial lawns and then shifted to 22°C for 4 h. For each assay, 15 to 20 L4-synchronized worms were added to the killing and control lawns and incubated at 22°C. Worm survival was scored at 4, 6, 8, 20, and 24 h after the start of the assay, using an Axiovert S100 optical microscope (Zeiss).

II.1.7. Bacterial cell viability assays by flow cytometry

Cell viability assays of *P. aeruginosa* H103 cells untreated and treated by plant extracts were assessed by using the LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit, for flow cytometry (Invitrogen, Molecular Probes). Briefly, *P. aeruginosa* H103 untreated or treated suspensions collected after 24 h of incubation with shaking (180 rpm) at 37 °C were washed twice and diluted in filter-sterilized PBS to reach a final density of 1×10^6 CFU/mL, then stained following the manufacturer's instructions. The data were acquired using CytoFlex S flow cytometer (Beckman coulter Life science). An aliquot of cells was killed with 100 % ethanol and used as a control of death cells. The SYTO9 stained cells were detected by an excitation with 22 mW blue laser at 488 and with emission wavelength at 525 nm (green, with band pass filter of 40 nm), while the PI (Propidium iodide) stained cells were detected by 690 nm (red, with band pass filter of 50 nm).

II.2. Molecular biology experimental procedures

II.2.1. Total RNA extraction

P. aeruginosa H103 untreated or plant extracts treated cultures were used to isolate total RNAs by the hot acid- phenol method (Bouffartigues *et al.*, 2012). Briefly, 300 μ L of the lysis buffer (0.5 % of SDS, 20 mM of sodium acetate, 1 mM of EDTA in RNase-free H₂O) was added to the cells pellets. Immediately, the lysates were added to Eppendorf tubes containing 600 μ L of phenol solution (saturated with 0.1 M citrate buffer, pH 4.3 ± 0.2) (Sigma Aldrich) and 100 μ L of RNase-free H₂O preheated

to 60 °C. The tubes were vortexed vigorously for 10 sec and incubated at 60 °C for 4 min. After centrifugation at 18,700 x g at RT for 4 min, the supernatant was carefully collected and extracted a second time with 600 µL of phenol solution and 100 µL of RNase-free H₂O. The tubes were centrifuged at 18,700 x g at RT for 4 min. The supernatant was collected and extracted with 1 volume of chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich) at RT to remove any remaining protein contamination and residual phenol. The collected supernatant was placed into a clean tube and 100 mM (1/10 volume) of sodium acetate (Sigma-Aldrich) and 2 volumes of ice-cold 100% ethanol (Sigma-Aldrich) were added to precipitate the RNAs. The tubes were placed in -20 °C freezer overnight. Next, the samples were centrifuged at 18,700 x g at 4 °C for 30 min. The liquid was carefully discarded and 1 mL of 70% ethanol was added to wash the RNA pellet. After centrifugation of the samples at 18,700 x g at 4 °C for 10 min, the liquid was carefully aspirated and the RNA pellets were allowed to air dry to remove all ethanol. The RNA pellets were resuspended in 50 µL of RNase- free water.

II.2.2. Removal of genomic DNA

Genomic DNA contamination was removed by using Turbo DNA-free™ kit (Invitrogen) according to the manufacturer's protocol. Briefly, a mixture of 43.5 µL of RNA template, 5 µL of 10X Turbo™ DNase buffer and 1.5 µL of Turbo™ DNase) was incubated for 45 min at 37 °C and the Turbo™ DNase was then inactivated by adding 1 µL of 0.5 M EDTA (pH 8.0) treated with DEPC (Sigma-Aldrich) to the solution and incubating for 10 min at 75 °C. Once RNA samples were confirmed genomic DNA-free by PCR, RNA samples were purified using Monarch® RNA Cleanup Kit (New England Biolabs) according to manufacturer's instructions. The concentration of RNAs was assayed using the NanoDrop® ND-1000

spectrophotometer (Thermo Scientific) and the quality was checked by running aliquots of RNA samples on a 2% agarose gel. Thus, the RNA samples of high quality and A260/A280 ratio \geq to 2 were then stored at -80 °C until used for cDNA synthesis.

II.2.3. cDNA synthesis

The extracted total RNAs were reverse transcribed into complementary DNA using High Capacity cDNA Reversed Transcription kit (Applied Biosystems, Foster City, California, USA) according to manufacturer's instructions. Briefly, cDNA synthesis was carried out in a final volume of 50 μ L containing 5 μ L of 10X RT-buffer, 5 μ L of 10X RT-primer, 2 μ L of 100 mM dNTPs, 2.5 μ L of RT-enzyme, 2 μ g of RNA and RNase free water to make up the final volume. The reaction was incubated for 2 h at 37 °C followed by inactivation of the reverse transcriptase at 85°C for 5 min. Control samples without the reverse transcriptase were performed under the same conditions.

II.2.4. Real time-quantitative PCR (RT-qPCR)

Quantitative PCR reactions were carried out using SYBR Green PCR Master Mix™ (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 Fast Q-PCR system (Applied Biosystems, Foster City, CA, USA). All primers used in this study were designed with Primer3 plus software (**Table 4**) and were chemically synthesized and purified by Eurogentec (Liège, Belgium). The mRNAs expression levels were normalized on 16S rRNA threshold cycle (Ct) values and calculated by comparing the Ct of targeted genes with those of control sample groups. The relative

quantification data were determined with the $2^{-\Delta\Delta C_t}$ method using DataAssist™ software (Applied Biosystems).

Table 4. List of primers used for quantification of mRNA levels by RT-qPCR

PA number	Gene name	Primer name	Sequence (5' > 3') ^a	Length
PA4210	<i>phzA</i>	<i>phzA-F</i>	AACCACTACATCCATTCCTTCG	22
		<i>phzA-R</i>	CGGCTATTCCCAATGCAC	18
PA0051	<i>phzH</i>	<i>phzH-F</i>	CGCGGGTTGGGTGGAT	16
		<i>phzH-R</i>	ATGACCGATACGCTCGCC	18
PA4209	<i>phzM</i>	<i>phzM-F</i>	GCTGCGCGTAATTTGATACAAG	22
		<i>phzM-R</i>	GATCCCGCTCTCGATCAGATC	21
PA4217	<i>phzS</i>	<i>phzS-F</i>	CCTGCGCGAATACGAAGAAG	20
		<i>phzS-R</i>	CGGCCCATTCCTCTTTTC	19
PA0996	<i>pqsA</i>	<i>pqsA-F</i>	CGGAGTTGCTGGCATTGC	18
		<i>pqsA-R</i>	CTGTTGCCCATGCCATAGC	19
PA2587	<i>pqsH</i>	<i>pqsH-F</i>	CTCCATCGTGCAGATCCT	18
		<i>pqsH-R</i>	CGGAATGACGCAAGGTC	17
PA4190	<i>pqsL</i>	<i>pqsL-F</i>	CGGTATCGCCTCCTACGTG	19
		<i>pqsL-R</i>	GGAAGCTCACCACCAGTCG	19
PA1003	<i>pqsR</i>	<i>pqsR-F</i>	AACCTGGAAATCGACCTGTG	20
		<i>pqsR-R</i>	TGAAATCGTCGAGCAGTACG	20
PA1776	<i>sigX</i>	<i>sigX-F</i>	AATTGATGCGGCGTTACCA	19
		<i>sigX-R</i>	CCAGGTAGCGGGCACAGA	18
PA3639	<i>accA</i>	<i>accA-F</i>	TCTTCGGCAATCTGACCAGTT	21
		<i>accA-R</i>	GTAGCCGATGTAGTCGAGGGTA	22
PA4847	<i>accB</i>	<i>accB-F</i>	AAGCCATGAAGATGATGAACC	21
		<i>accB-R</i>	CGTTCTCCACCAGGATCGA	19
PA5174	<i>fabY</i>	<i>fabY-F</i>	AGGGCGACCTGGAGATCAT	19
		<i>fabY-R</i>	GCGCGTCCTTCTTGTATACCA	21
	16S	<i>16S-F</i>	AACCTGGGAACTGCATCCAA	20
		<i>16S-R</i>	CTTCGCCACTGGTGTTCCTT	20

^a All the primers used in this study were synthesized by Eurogentec and are based on *P. aeruginosa* PAO1 genome sequence (<http://www.pseudomonas.com>).

II.2.5. Transcriptional fusion *PsigX::luxCDABE* construction

The promoter region of the *sigX* gene (*PsigX*) was amplified by PCR with primers *PsigX-SacI-F* (5'-taataa-GAGCTC-gagtcgctcggcctgca-3') and *PsigX-SpeI-R* (5'-taataaa-CTAGTG-gtggaacagctccgagtgcg-3') incorporating *SacI* and *SpeI* linkers

highlighted in bold, respectively. The promoter region of *sigX* gene was fused to the *luxCDABE* cassette in the promoterless pAB133 vector (Gm^R) (see **Table 2**) (Bazire et., 2010). Upon amplification, DNA was digested with the appropriate restriction enzymes, and cloned into pAB133 generating pAB-P*sigX* vector (see **Table 2**). The pAB-P*sigX* plasmid was then transformed separately into one shot™ *E. coli* TOP10 competent cells (Invitrogen). The constructions were confirmed by DNA sequencing (Sanger sequencing services, Genewiz). Finally, the plasmids were transferred by electroporation into *P. aeruginosa* H103 strain.

II.3. Biochemical assays

II.3.1. Detection of AHL- and HAQs-quorum sensing molecules

To detect AHLs, an overnight culture of the AHL indicator strain *Chromobacterium violaceum* CV026 was diluted 1:100 in 5 mL LB medium and poured onto LB agar. Once the plates were dried, paper disks of 5 mm in diameter were placed on an agar plate and the AHL samples were applied. The assay plates were incubated overnight at 28 °C and the appearance of violacein pigment around the filter was determined. HAQs QS molecules were detected using the biosensor strain *Pseudomonas aeruginosa* PAO1 $\Delta pqsA$ CTX-*lux::pqsA* as previously described (Fletcher et al., 2007; Tahrioui et al., 2011) using a combined spectrophotometer/luminometer microplate assay. Briefly, 5 μ L of the crude HAQ extracts obtained from *P. aeruginosa* H103 untreated or plant extracts treated conditions were diluted in LB medium. Aliquots of 100 μ L of this dilution were mixed with 100 μ L of 1:50 dilution of PAO1 $\Delta pqsA$ CTX-*lux::pqsA* biosensor. Further, bioluminescence and O.D at 580 nm were monitored in specialized white sided and clear bottom 96-well microtiter plate (Perkin Elmer, Waltham, MA) every 15 min at

37 °C for 24 h using the Spark 20M multimode Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland) controlled by SparkControl™ software Version 2.1. Both HHQ and PQS synthetic standards (Sigma-Aldrich) prepared at 5 µM in 1:100 dilution of the HAQ biosensor strain served as positive controls whereas the same dilution of biosensor strain served as a negative control. The activity of the reporter strain was quantified as R.L.U (Relative Luminescence Unit)/O.D_{580nm} for each culture suspension.

II.3.2. *sigX* promoter activity analysis

For bioluminescence assays, *P. aeruginosa* H103 strains harboring pAB-PsigX reporter vector and pAB133 promoter-less vector (see **Table 2**) were grown in LB medium supplemented with gentamycin at 50 µg/mL. Then, untreated and treated cultures were prepared from an initial inoculum adjusted to an OD_{580nm} value of 0.08 in LB (without gentamycin) in specialized white sided 96-well microtiter plates with a flat transparent bottom (Nunc™ MicroWell™, Thermo Scientific™). Bioluminescence and growth (OD_{580nm}) were monitored every 15 min for 24 h at 37 °C with shaking (180 rpm) using the Spark 20M multimode Microplate Reader controlled by SparkControl™ software Version 2.1 (Tecan Group Ltd.). The relative light units (R.L.U) of the negative control strain (*P. aeruginosa* harboring the promoter-less vector pAB133) were subtracted from those containing the studied promoter. The bioluminescence values in relative light units (R.L.U) and Log(O.D_{580nm}) values were plotted.

II.3.3. Fluorescence anisotropy measurements

Membrane fluidity analysis of *P. aeruginosa* H103 wild-type and $\Delta sigX$ mutant untreated or treated cultures with plant extracts and fractions was performed via fluorescence anisotropy (Flécharde *et al.*, 2018). Bacterial cells were centrifuged (7,500 x g for 10 min at 25 °C). Pellets were washed twice with 10 mM MgSO₄ before resuspension in same wash solution to reach an O.D value of 0.1 at 580 nm. Then, 1 µL of 4 mM DPH (1,6-diphenyl 1,3,5 hexatriene) dissolved in tetrahydrofuran was added to 1 mL aliquot of the diluted suspension and incubated at 37 °C for 30 min in the dark to allow the incorporation of the probe into the lipid membranes. Subsequently, fluorescence polarization was measured the Spark 20M multimode Microplate Reader, equipped with an active temperature regulation system (Te-Cool™, Tecan Group Ltd., Switzerland) with wavelengths of emission and excitation set at 425 nm and 365 nm, respectively. Calculation of fluorescence anisotropy (FA) values was performed according to following formula (Lakowicz, 2010):

$$r = \frac{(I1 - I2)}{(I1 + 2xGI2)}$$

(I1) correspond to the emission fluorescence intensity measured alongside and (I2) correspond to the emission fluorescence intensity measured perpendicularly to light excitation plan. G correspond to G factor. The relationship between anisotropy and membrane fluidity is an inverse one, where decreasing anisotropy values correspond to a more fluid membrane and *vice versa*.

II.3.4. Cytotoxicity assessment on A549 human pulmonary cell

The LDH release assays were also used to determine the cytotoxicity of plant extract or fractions in A549 cells upon direct exposure after 1, 3, 6, and 24 h

incubation. Once A549 cells reach confluency status on 24-well microtiter plate, the wells were replenished with 900 μ L of DMEM medium per well. Then, 100 μ L aliquots of plant extracts or fractions at the required concentration were directly added to 900 μ L of cell culture suspension with subsequent incubation of plate at 37°C in an enriched atmosphere with 5% CO₂. The corresponding negative controls of 1% DMSO, DMEM media alone and lysis buffer as positive control were prepared. Toxicity of plant extract or fractions was assessed enzymatically at 1, 3, 6, 24 h post-inoculation using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific™) as recommended by the manufacturer.

II.4. Chemical analyses

II.4.1. Plant extracts preparation

Samples were extracted at Laboratoire de pharmacognosie CNRS-UMR 8638, Université Paris Descartes Sorbonne Paris Cité. First, the plants were dried under air, then pulverized using an electric grinder pulverisette 19 (Fritsch GmbH, Mahlen und Messen, Germany). Approximately 20g of plant powder were subjected to pressurized solvent extraction (PSE), extraction according to the medicinal purpose previously described for each part of plant. The Speed extractor E-914 (Büchi, Fawil Switzerland) equipped with four cells (120 mL) and a flat bottom vial collector (220 mL) was set at two extraction cycles with fifteen minutes hold on time at 100 bar maximum pressure and 50 °C temperature. Ethyl acetate (AcOEt) and methanol (MeOH) were used as solvents. The solvents were removed under reduced pressure with a rotavapor and extracts were labelled with different codes. Subsequently, extracts were solubilized at 10 mg/mL in 100 % DMSO and stored at -20 °C prior to biological evaluation.

II.4.2. Plant extracts fractionation

The fractionation of active extracts were done with silica gel column chromatography using a combination of dichloromethane and methanol as solvent. The active fractions were subjected to a second silica gel chromatography using gradient of petroleum ether and ethyl acetate or dichloromethane and methanol mixtures as solvents.

II.4.3. Phytochemical profiling and molecular networks construction

II.4.3.1. HRMS analysis

Chromatographic separation was performed on a Waters Acquity UPLC system hyphenated to a Q-Exactive Focus mass spectrometer (Thermo Scientific, Bremen, Germany), using a heated electrospray ionization (HESI-II) source and a CAD detector (Thermo Scientific, Bremen, Germany). The instrument was controlled using Thermo Scientific Xcalibur 3.1 software. The LC separation was done on a Waters BEH C18 50 × 2.1 mm, 1.7 μm column, using a linear gradient of 5–100 % B over 7 min and an isocratic step at 100 % B for 1 min. Mobile phase, (A) water with 0.1 % formic acid; (B) acetonitrile with 0.1 % formic acid. All analyses with flow rate, 600 μL/min and an injection volume of 2 μL. ESI parameters were as follows: source voltage, 3.5 kV (pos); sheath gas flow rate (N₂), 55 units; auxiliary gas flow rate, 15 units; spare gas flow rate, 3.0; capillary temperature, 350.00°C, S-Lens RF Level, 45. The mass analyzer was calibrated using a mixture of caffeine, methionine–arginine–phenylalanine–alanine–acetate (MRFA), sodium dodecyl sulfate, sodium

taurocholate, and Ultramark 1621 in an acetonitrile/methanol/water solution containing 1 % formic acid by direct injection. The data-dependent MS/MS events were performed on the three most intense ions detected in full scan MS (Top3 experiment). The MS/MS isolation window width was 1 Da, and the stepped normalized collision energy (NCE) was set to 15, 30 and 45 units. In data-dependent MS/MS experiments, full scans were acquired at a resolution of 35,000 FWHM (at m/z 200) and MS/MS scans at 17,500 FWHM both with an automatically determined maximum injection time. After being acquired in a MS/MS scan, parent ions were placed in a dynamic exclusion list for 2.0 s.

II.4.3.2. MS Data treatment

All HRMS runs data was converted from .RAW (Thermo) standard data format to .mzXML format using the MS Convert software, part of the ProteoWizard package (Holman *et al.*, 2014). The converted files were treated using the MZmine software suite version 2.53 (Pluskal *et al.*, 2010). For mass detection at MS¹ level the noise level was set to 1.0E4 for positive mode and 1.0E5 for negative mode. For MS² detection the noise level was set to 0.0E0 was set for both ionization modes. The ADAP chromatogram builder was used and set to a minimum group size of scans of 5, minimum group intensity threshold of 1.0E6 (1.0E5 negative), minimum highest intensity of 1.0E6 (1.0E5 negative) and m/z tolerance of 8.0 ppm. ADAP algorithm (wavelets) was used for chromatogram deconvolution. The intensity window S/N was used as S/N estimator with a signal to noise ratio set at 25, a minimum feature height at 1.0E6 (1.0E5 negative), a coefficient area threshold at 100, a peak duration ranges from 0.02 to 1.0 min and the RT wavelet range from 0.02 to 0.08 min. Isotopes were detected using the isotopes peaks grouper with a m/z tolerance of 5.0 ppm, a RT tolerance of 0.02 min (absolute), the maximum charge set at 1 and the representative isotope used was the most intense. The peak list was gap-

filled with the same RT and m/z range gap filler (m/z tolerance at 8 ppm). Eventually the resulting aligned peak list was filtered using the peak-list rows filter option to remove all the duplicates and all the features without a MS² spectrum associated.

II.4.3.3. Mass spectral organization (Molecular Networks) and taxonomically informed metabolite annotation.

A molecular network was constructed from the mgf file exported from MZmine, using the online workflow (<https://ccms-ucsd.github.io/GNPSDocumentation/>) on the GNPS website (<http://gnps.ucsd.edu>) (Wang *et al.*, 2016). The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 3 matched peaks. The works are available in the following links, negative: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c0e684e7e3d7493d9d99501f75b18b14>. Positive: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c8a10978dc7d4879b08c21bec7c93a1a>. Combined network extract and fractions in negative mode: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=839e6bd755e44fa2aae90901b62c527b>.

Data visualization was achieved using Cytoscape version 3.8.0 (Shannon *et al.*, 2003). The output of the GNPS was used to annotate against the in silico ISDB-DNP (Allard *et al.*, 2016) and then the script for taxonomically informed metabolite annotation (Rutz *et al.*, 2019) was used to re rank and clean the output based on the species.

II.4.5. Structure determination

The structural identification of derived compounds was ascertained and ^1D , ^2D NMR in deuterated chloroform (CDCl_3) and mass spectrophotometry (ZQ 2000 Waters Saint -Quentin, France), then compared with published data. The spectra arising from the isolated pure compounds were recorded on a spectrometer (Bruker AC 300MHz or Bruker AC 400MHz, Wissembourg, France) whereas the rotary values were made on a polarimeter at 20 °C (Perkin Elmer model 341, Perkin Elmer Courtabouef, France).

II.4.4. Gas chromatography-mass spectrometry (GC-MS) analyses

The GC-MS analyses were carried out in a Hewlett Packard GC 6890/MSD 5972 apparatus equipped with HP-5 (30 m x 0.25 mm x 0.25 μm). Carried gas was Ar with a flow of 0.9 mL/min, split 1:20, oven was programmed increasing from 180 °C to 270 °C at 8°C min⁻¹ with an initial and final hold of 1 and 65 min, respectively. The inlet and GC-MS interface temperature were kept at 240 °C. The temperature of EI (electron impact) 70 eV was 220 °C with full scan (80-500 m/z). The injection volume was 1 μL . Identification of constituents was achieved by comparing their mass spectra with those of the Mass Spectra Library (NIST 98) compounds.

II.4.5. Extraction of crude quorum sensing (QS) molecules

Whole cultures of *P. aeruginosa* H103 untreated or plant extracts treated were extracted twice with equal volumes of ethyl acetate acidified with glacial acetic acid

at 0.01 %. The collected organic layer were evaporated to dryness under a chemical hood. The crude QS molecules extracts were resuspended in 70 % (vol/vol) methanol prior to their detection by biosensor strains.

II.5. Statistical analyses

All experiments were performed at least in triplicate measurements of three independent assays. Means and standard error of the means were calculated and plotted. Statistical data analyses were carried out with Graph Pad prism online calculator using (two samples) two-tailed *t*-test. *C. elegans* survival curves were prepared using GraphPad Prism 8 to perform a statistical log-rank (Mantel-Cox) test. Significance was considered at ****, $P < 0.0001$; ***, $P = 0.0001$ to 0.001 ; **, $P = 0.001$ to 0.01 ; *, $P = 0.01$ to 0.05 ; NS (Not Significant), $P > 0.05$.

REFERENCES

- Abdel-Mawgoud, A.M., Aboulwafa, M.M., and Hassouna, N.A. (2009). Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Appl Biochem Biotechnol* 157(2), 329-345. doi: 10.1007/s12010-008-8285-1.
- Acosta, N., Waddell, B., Heirali, A., Somayaji, R., Surette, M.G., Workentine, M.L., et al. (2020). Cystic Fibrosis Patients Infected with Epidemic *Pseudomonas aeruginosa* Strains Have Unique Microbial Communities. *Front Cell Infect Microbiol* 10, 173. doi: 10.3389/fcimb.2020.00173.
- Adam, E.C., Mitchell, B.S., Schumacher, D.U., Grant, G., and Schumacher, U. (1997). *Pseudomonas aeruginosa* II lectin stops human ciliary beating: therapeutic implications of fucose. *Am J Respir Crit Care Med* 155(6), 2102-2104. doi: 10.1164/ajrccm.155.6.9196121.
- Ahmad, S., Sukari, M.A., Ismail, N., Ismail, I.S., Abdul, A.B., Abu Bakar, M.F., et al. (2015). Phytochemicals from *Mangifera pajang* Kosterm and their biological activities. *BMC Complement Altern Med* 15, 83. doi: 10.1186/s12906-015-0594-7.
- Ahmad, V., Jamal, Q.M.S., Siddiqui, M.U., Shukla, A.K., Alzohairy, M.A., Al Karaawi, M.A., et al. (2017). Methods of Screening-Purification and Antimicrobial Potentialities of Bacteriocin in Health Care. *Curr Drug Metab* 18(9), 814-830. doi: 10.2174/1389200218666170116111309.
- Albus, A.M., Pesci, E.C., Runyen-Janecky, L.J., West, S.E., and Iglewski, B.H. (1997). Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179(12), 3928-3935. doi: 10.1128/jb.179.12.3928-3935.1997.
- Alcalde-Rico, M., Olivares-Pacheco, J., Halliday, N., Camara, M., and Martinez, J.L. (2020). The impaired quorum sensing response of *Pseudomonas aeruginosa* MexAB-OprM efflux pump overexpressing mutants is not due to non-physiological efflux of 3-oxo-C12-HSL. *Environ Microbiol*. doi: 10.1111/1462-2920.15177.
- Alhede, M., Bjarnsholt, T., Jensen, P.O., Phipps, R.K., Moser, C., Christophersen, L., et al. (2009). *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. *Microbiology (Reading)* 155(Pt 11), 3500-3508. doi: 10.1099/mic.0.031443-0.
- Allen, L., Dockrell, D.H., Pattery, T., Lee, D.G., Cornelis, P., Hellewell, P.G., et al. (2005). Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. *J Immunol* 174(6), 3643-3649. doi: 10.4049/jimmunol.174.6.3643.
- Almblad, H., Harrison, J.J., Rybtke, M., Groizeleau, J., Givskov, M., Parsek, M.R., et al. (2015). The Cyclic AMP-Vfr Signaling Pathway in *Pseudomonas aeruginosa* Is Inhibited by Cyclic Di-GMP. *J Bacteriol* 197(13), 2190-2200. doi: 10.1128/JB.00193-15.
- Andrews, S.C., Robinson, A.K., and Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* 27(2-3), 215-237. doi: 10.1016/S0168-6445(03)00055-X.
- Anwar, H., and Costerton, J.W. (1990). Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 34(9), 1666-1671. doi: 10.1128/aac.34.9.1666.
- Asfahl, K.L., and Schuster, M. (2017). Additive Effects of Quorum Sensing Anti-Activators on *Pseudomonas aeruginosa* Virulence Traits and Transcriptome. *Front Microbiol* 8, 2654. doi: 10.3389/fmicb.2017.02654.
- Bainbridge, T., and Fick, R.B., Jr. (1989). Functional importance of cystic fibrosis immunoglobulin G fragments generated by *Pseudomonas aeruginosa* elastase. *J Lab Clin Med* 114(6), 728-733.

- Bajolet-Laudinat, O., Girod-de Bentzmann, S., Tournier, J.M., Madoulet, C., Plotkowski, M.C., Chippaux, C., et al. (1994). Cytotoxicity of *Pseudomonas aeruginosa* internal lectin PA-I to respiratory epithelial cells in primary culture. *Infect Immun* 62(10), 4481-4487. doi: 10.1128/IAI.62.10.4481-4487.1994.
- Banin, E., Vasil, M.L., and Greenberg, E.P. (2005). Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A* 102(31), 11076-11081. doi: 10.1073/pnas.0504266102.
- Barbieri, J.T., and Sun, J. (2004). *Pseudomonas aeruginosa* ExoS and ExoT. *Rev Physiol Biochem Pharmacol* 152, 79-92. doi: 10.1007/s10254-004-0031-7.
- Barken, K.B., Pamp, S.J., Yang, L., Gjermansen, M., Bertrand, J.J., Klausen, M., et al. (2008). Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol* 10(9), 2331-2343. doi: 10.1111/j.1462-2920.2008.01658.x.
- Becerra, C., Albasa, I., and Eraso, A.J. (2001). Leukotoxicity of pyoverdinin, production of reactive oxygen species, and effect of UV radiation. *Biochem Biophys Res Commun* 285(2), 414-418. doi: 10.1006/bbrc.2001.5188.
- Beckmann, B.E., Knoester, D.B., Connelly, B.D., Waters, C.M., and McKinley, P.K. (2012). Evolution of resistance to quorum quenching in digital organisms. *Artif Life* 18(3), 291-310. doi: 10.1162/artl_a_00066.
- Ben Haj Khalifa, A., Moissenet, D., Vu Thien, H., and Khedher, M. (2011). [Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation]. *Ann Biol Clin (Paris)* 69(4), 393-403. doi: 10.1684/abc.2011.0589.
- Blanka, A., Schulz, S., Eckweiler, D., Franke, R., Bielecka, A., Nicolai, T., et al. (2014). Identification of the alternative sigma factor SigX regulon and its implications for *Pseudomonas aeruginosa* pathogenicity. *J Bacteriol* 196(2), 345-356. doi: 10.1128/JB.01034-13.
- Bleves, S., Viarre, V., Salacha, R., Michel, G.P., Filloux, A., and Voulhoux, R. (2010). Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. *Int J Med Microbiol* 300(8), 534-543. doi: 10.1016/j.ijmm.2010.08.005.
- Boechat, A.L., Kaihama, G.H., Politi, M.J., Lepine, F., and Baldini, R.L. (2013). A novel role for an ECF sigma factor in fatty acid biosynthesis and membrane fluidity in *Pseudomonas aeruginosa*. *PLoS One* 8(12), e84775. doi: 10.1371/journal.pone.0084775.
- Boles, B.R., Thoendel, M., and Singh, P.K. (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol Microbiol* 57(5), 1210-1223. doi: 10.1111/j.1365-2958.2005.04743.x.
- Bollinger, N., Hassett, D.J., Iglewski, B.H., Costerton, J.W., and McDermott, T.R. (2001). Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J Bacteriol* 183(6), 1990-1996. doi: 10.1128/JB.183.6.1990-1996.2001.
- Borchardt, S.A., Allain, E.J., Michels, J.J., Stearns, G.W., Kelly, R.F., and McCoy, W.F. (2001). Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. *Appl Environ Microbiol* 67(7), 3174-3179. doi: 10.1128/AEM.67.7.3174-3179.2001.
- Borlee, B.R., Goldman, A.D., Murakami, K., Samudrala, R., Wozniak, D.J., and Parsek, M.R. (2010). *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol Microbiol* 75(4), 827-842. doi: 10.1111/j.1365-2958.2009.06991.x.

- Borquez, J., Ardiles, A., Loyola, L.A., Pena-Rodriguez, L.M., Molina-Salinas, G.M., Vallejos, J., et al. (2014). Further mulinane and azorellane diterpenoids isolated from *Mulinum crassifolium* and *Azorella compacta*. *Molecules* 19(4), 3898-3908. doi: 10.3390/molecules19043898.
- Borris, R.P. (1996). Natural products research: perspectives from a major pharmaceutical company. *J Ethnopharmacol* 51(1-3), 29-38. doi: 10.1016/0378-8741(95)01347-4.
- Bouffartigues, E., Gicquel, G., Bazire, A., Bains, M., Maillot, O., Vieillard, J., et al. (2012). Transcription of the *oprF* gene of *Pseudomonas aeruginosa* is dependent mainly on the SigX sigma factor and is sucrose induced. *J Bacteriol* 194(16), 4301-4311. doi: 10.1128/JB.00509-12.
- Bouffartigues, E., I., H.M., Maillot, O., Tortuel, D., Omnes, J., David, A., et al. (2020). The temperature-regulation of *Pseudomonas aeruginosa* *cmaX-cfrX-cmpX* operon reveals an intriguing molecular network involving the sigma factors AlgU and SigX. *Front Microbiol* 11.
- Bozorgi, M., Memariani, Z., Mobli, M., Salehi Surmaghi, M.H., Shams-Ardekani, M.R., and Rahimi, R. (2013). Five *Pistacia* species (*P. vera*, *P. atlantica*, *P. terebinthus*, *P. khinjuk*, and *P. lentiscus*): a review of their traditional uses, phytochemistry, and pharmacology. *ScientificWorldJournal* 2013, 219815. doi: 10.1155/2013/219815.
- Branny, P., Pearson, J.P., Pesci, E.C., Kohler, T., Iglewski, B.H., and Van Delden, C. (2001). Inhibition of quorum sensing by a *Pseudomonas aeruginosa* *dksA* homologue. *J Bacteriol* 183(5), 1531-1539. doi: 10.1128/JB.183.5.1531-1539.2001.
- Breidenstein, E.B., de la Fuente-Nunez, C., and Hancock, R.E. (2011). *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 19(8), 419-426. doi: 10.1016/j.tim.2011.04.005.
- Brencic, A., and Lory, S. (2009). Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72(3), 612-632. doi: 10.1111/j.1365-2958.2009.06670.x.
- Britigan, B.E., Railsback, M.A., and Cox, C.D. (1999). The *Pseudomonas aeruginosa* secretory product pyocyanin inactivates alpha1 protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease. *Infect Immun* 67(3), 1207-1212. doi: 10.1128/IAI.67.3.1207-1212.1999.
- Britigan, B.E., Roeder, T.L., Rasmussen, G.T., Shasby, D.M., McCormick, M.L., and Cox, C.D. (1992). Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas*-associated tissue injury. *J Clin Invest* 90(6), 2187-2196. doi: 10.1172/JCI116104.
- Burrowes, E., Baysse, C., Adams, C., and O'Gara, F. (2006). Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. *Microbiology (Reading)* 152(Pt 2), 405-418. doi: 10.1099/mic.0.28324-0.
- Caiazza, N.C., Shanks, R.M., and O'Toole, G.A. (2005). Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol* 187(21), 7351-7361. doi: 10.1128/JB.187.21.7351-7361.2005.
- Calfee, M.W., Shelton, J.G., McCubrey, J.A., and Pesci, E.C. (2005). Solubility and bioactivity of the *Pseudomonas* quinolone signal are increased by a *Pseudomonas aeruginosa*-produced surfactant. *Infect Immun* 73(2), 878-882. doi: 10.1128/IAI.73.2.878-882.2005.
- Cegelski, L., Marshall, G.R., Eldridge, G.R., and Hultgren, S.J. (2008). The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* 6(1), 17-27. doi: 10.1038/nrmicro1818.
- Cezairliyan, B., Vinayavekhin, N., Grenfell-Lee, D., Yuen, G.J., Saghatelian, A., and Ausubel, F.M. (2013). Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. *PLoS Pathog* 9(1), e1003101. doi: 10.1371/journal.ppat.1003101.

- Chambonnier, G., Roux, L., Redelberger, D., Fadel, F., Filloux, A., Sivaneson, M., et al. (2016). The Hybrid Histidine Kinase LadS Forms a Multicomponent Signal Transduction System with the GacS/GacA Two-Component System in *Pseudomonas aeruginosa*. *PLoS Genet* 12(5), e1006032. doi: 10.1371/journal.pgen.1006032.
- Chemani, C., Imberty, A., de Bentzmann, S., Pierre, M., Wimmerova, M., Guery, B.P., et al. (2009). Role of LecA and LecB lectins in *Pseudomonas aeruginosa*-induced lung injury and effect of carbohydrate ligands. *Infect Immun* 77(5), 2065-2075. doi: 10.1128/IAI.01204-08.
- Chen, F., Gao, Y., Chen, X., Yu, Z., and Li, X. (2013). Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection. *Int J Mol Sci* 14(9), 17477-17500. doi: 10.3390/ijms140917477.
- Chevalier, S., Bouffartigues, E., Bazire, A., Tahrioui, A., Duchesne, R., Tortuel, D., et al. (2019). Extracytoplasmic function sigma factors in *Pseudomonas aeruginosa*. *Biochim Biophys Acta Gene Regul Mech* 1862(7), 706-721. doi: 10.1016/j.bbagr.2018.04.008.
- Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., and Greenberg, E.P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 98(5), 2752-2757. doi: 10.1073/pnas.051624298.
- Ciofu, O., Mandsberg, L.F., Wang, H., and Hoiby, N. (2012). Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of *Pseudomonas aeruginosa* biofilm infections. *FEMS Immunol Med Microbiol* 65(2), 215-225. doi: 10.1111/j.1574-695X.2012.00983.x.
- Coggan, K.A., and Wolfgang, M.C. (2012). Global regulatory pathways and cross-talk control *Pseudomonas aeruginosa* environmental lifestyle and virulence phenotype. *Curr Issues Mol Biol* 14(2), 47-70.
- Collier, D.N., Anderson, L., McKnight, S.L., Noah, T.L., Knowles, M., Boucher, R., et al. (2002). A bacterial cell to cell signal in the lungs of cystic fibrosis patients. *FEMS Microbiol Lett* 215(1), 41-46. doi: 10.1111/j.1574-6968.2002.tb11367.x.
- Colvin, K.M., Gordon, V.D., Murakami, K., Borlee, B.R., Wozniak, D.J., Wong, G.C., et al. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog* 7(1), e1001264. doi: 10.1371/journal.ppat.1001264.
- Comolli, J.C., Hauser, A.R., Waite, L., Whitchurch, C.B., Mattick, J.S., and Engel, J.N. (1999). *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. *Infect Immun* 67(7), 3625-3630. doi: 10.1128/IAI.67.7.3625-3630.1999.
- Cooke, A.C., Nello, A.V., Ernst, R.K., and Schertzer, J.W. (2019). Analysis of *Pseudomonas aeruginosa* biofilm membrane vesicles supports multiple mechanisms of biogenesis. *PLoS One* 14(2), e0212275. doi: 10.1371/journal.pone.0212275.
- Cornelis, P., and Dingemans, J. (2013). *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. *Front Cell Infect Microbiol* 3, 75. doi: 10.3389/fcimb.2013.00075.
- Cornelis, P., Matthijs, S., and Van Oeffelen, L. (2009). Iron uptake regulation in *Pseudomonas aeruginosa*. *Biometals* 22(1), 15-22. doi: 10.1007/s10534-008-9193-0.
- Crabbe, A., De Boever, P., Van Houdt, R., Moors, H., Mergeay, M., and Cornelis, P. (2008). Use of the rotating wall vessel technology to study the effect of shear stress on growth behaviour of *Pseudomonas aeruginosa* PA01. *Environ Microbiol* 10(8), 2098-2110. doi: 10.1111/j.1462-2920.2008.01631.x.

- Crabbe, A., Pycke, B., Van Houdt, R., Monsieurs, P., Nickerson, C., Leys, N., et al. (2010). Response of *Pseudomonas aeruginosa* PAO1 to low shear modelled microgravity involves AlgU regulation. *Environ Microbiol* 12(6), 1545-1564. doi: 10.1111/j.1462-2920.2010.02184.x.
- Cragg, G.M., Newman, D.J., and Snader, K.M. (1997). Natural products in drug discovery and development. *J Nat Prod* 60(1), 52-60. doi: 10.1021/np9604893.
- Crone, S., Vives-Florez, M., Kvich, L., Saunders, A.M., Malone, M., Nicolaisen, M.H., et al. (2020). The environmental occurrence of *Pseudomonas aeruginosa*. *APMIS* 128(3), 220-231. doi: 10.1111/apm.13010.
- Cunrath, O., Gasser, V., Hoegy, F., Reimmann, C., Guillon, L., and Schalk, I.J. (2015). A cell biological view of the siderophore pyochelin iron uptake pathway in *Pseudomonas aeruginosa*. *Environ Microbiol* 17(1), 171-185. doi: 10.1111/1462-2920.12544.
- Davey, M.E., Caiazza, N.C., and O'Toole, G.A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185(3), 1027-1036. doi: 10.1128/jb.185.3.1027-1036.2003.
- Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280(5361), 295-298. doi: 10.1126/science.280.5361.295.
- Defoirdt, T. (2018). Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends Microbiol* 26(4), 313-328. doi: 10.1016/j.tim.2017.10.005.
- Deziel, E., Comeau, Y., and Villemur, R. (2001). Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpilated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* 183(4), 1195-1204. doi: 10.1128/JB.183.4.1195-1204.2001.
- Deziel, E., Gopalan, S., Tampakaki, A.P., Lepine, F., Padfield, K.E., Saucier, M., et al. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-L-homoserine lactones. *Mol Microbiol* 55(4), 998-1014. doi: 10.1111/j.1365-2958.2004.04448.x.
- Dietrich, L.E., Price-Whelan, A., Petersen, A., Whiteley, M., and Newman, D.K. (2006). The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* 61(5), 1308-1321. doi: 10.1111/j.1365-2958.2006.05306.x.
- Diggle, S.P., Fletcher, M.P., Camara, M., and Williams, P. (2011). Detection of 2-alkyl-4-quinolones using biosensors. *Methods Mol Biol* 692, 21-30. doi: 10.1007/978-1-60761-971-0_2.
- Diggle, S.P., Matthijs, S., Wright, V.J., Fletcher, M.P., Chhabra, S.R., Lamont, I.L., et al. (2007). The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 14(1), 87-96. doi: 10.1016/j.chembiol.2006.11.014.
- Diggle, S.P., Stacey, R.E., Dodd, C., Camara, M., Williams, P., and Winzer, K. (2006). The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*. *Environ Microbiol* 8(6), 1095-1104. doi: 10.1111/j.1462-2920.2006.001001.x.
- Diggle, S.P., Winzer, K., Chhabra, S.R., Worrall, K.E., Camara, M., and Williams, P. (2003). The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* 50(1), 29-43. doi: 10.1046/j.1365-2958.2003.03672.x.

- Diggle, S.P., Winzer, K., Lazdunski, A., Williams, P., and Camara, M. (2002). Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of *N*-acylhomoserine lactone production and virulence gene expression. *J Bacteriol* 184(10), 2576-2586. doi: 10.1128/jb.184.10.2576-2586.2002.
- Ding, F., Oinuma, K.I., Smalley, N.E., Schaefer, A.L., Hamwy, O., Greenberg, E.P., et al. (2018). The *Pseudomonas aeruginosa* Orphan Quorum Sensing Signal Receptor QscR Regulates Global Quorum Sensing Gene Expression by Activating a Single Linked Operon. *mBio* 9(4). doi: 10.1128/mBio.01274-18.
- Dixon, R.A. (2001). Natural products and plant disease resistance. *Nature* 411(6839), 843-847. doi: 10.1038/35081178.
- Duchesne, R., Bouffartigues, E., Oxaran, V., Maillot, O., Benard, M., Feuilloley, M.G., et al. (2013). A proteomic approach of SigX function in *Pseudomonas aeruginosa* outer membrane composition. *J Proteomics* 94, 451-459. doi: 10.1016/j.jprot.2013.10.022.
- Duong, F., Bonnet, E., Geli, V., Lazdunski, A., Murgier, M., and Filloux, A. (2001). The AprX protein of *Pseudomonas aeruginosa*: a new substrate for the Apr type I secretion system. *Gene* 262(1-2), 147-153. doi: 10.1016/s0378-1119(00)00541-2.
- Dzul-Beh, A.J., Uc-Cachon, A.H., Borquez, J., Loyola, L.A., Pena-Rodriguez, L.M., and Molina-Salinas, G.M. (2020). Mulinane- and Azorellane-Type Diterpenoids: A Systematic Review of Their Biosynthesis, Chemistry, and Pharmacology. *Biomolecules* 10(9). doi: 10.3390/biom10091333.
- El-Shaer, S., Shaaban, M., Barwa, R., and Hassan, R. (2016). Control of quorum sensing and virulence factors of *Pseudomonas aeruginosa* using phenylalanine arginyl beta-naphthylamide. *J Med Microbiol* 65(10), 1194-1204. doi: 10.1099/jmm.0.000327.
- Engel, J., and Balachandran, P. (2009). Role of *Pseudomonas aeruginosa* type III effectors in disease. *Curr Opin Microbiol* 12(1), 61-66. doi: 10.1016/j.mib.2008.12.007.
- Essar, D.W., Eberly, L., Hadero, A., and Crawford, I.P. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* 172(2), 884-900. doi: 10.1128/jb.172.2.884-900.1990.
- Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J., and Poole, K. (1998). Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 180(20), 5443-5447. doi: 10.1128/JB.180.20.5443-5447.1998.
- Fan, H., Dong, Y., Wu, D., Bowler, M.W., Zhang, L., and Song, H. (2013). QsIA disrupts LasR dimerization in antiactivation of bacterial quorum sensing. *Proc Natl Acad Sci U S A* 110(51), 20765-20770. doi: 10.1073/pnas.1314415110.
- Farrow, J.M., 3rd, and Pesci, E.C. (2007). Two distinct pathways supply anthranilate as a precursor of the *Pseudomonas* quinolone signal. *J Bacteriol* 189(9), 3425-3433. doi: 10.1128/JB.00209-07.
- Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., et al. (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* 66(1), 43-51. doi: 10.1128/IAI.66.1.43-51.1998.
- Feliziani, S., Lujan, A.M., Moyano, A.J., Sola, C., Bocco, J.L., Montanaro, P., et al. (2010). Mucoïdy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. *PLoS One* 5(9). doi: 10.1371/journal.pone.0012669.
- Feng, L., Rutherford, S.T., Papenfort, K., Bagert, J.D., van Kessel, J.C., Tirrell, D.A., et al. (2015). A *qrr* noncoding RNA deploys four different regulatory mechanisms to optimize quorum-sensing dynamics. *Cell* 160(1-2), 228-240. doi: 10.1016/j.cell.2014.11.051.

- Filloux, A. (2011). Protein Secretion Systems in *Pseudomonas aeruginosa*: An Essay on Diversity, Evolution, and Function. *Front Microbiol* 2, 155. doi: 10.3389/fmicb.2011.00155.
- Flechard, M., Duchesne, R., Tahrioui, A., Bouffartigues, E., Depayras, S., Hardouin, J., et al. (2018). The absence of SigX results in impaired carbon metabolism and membrane fluidity in *Pseudomonas aeruginosa*. *Sci Rep* 8(1), 17212. doi: 10.1038/s41598-018-35503-3.
- Flemming, H.C., and Wingender, J. (2010). The biofilm matrix. *Nat Rev Microbiol* 8(9), 623-633. doi: 10.1038/nrmicro2415.
- Fletcher, M.P., Diggle, S.P., Camara, M., and Williams, P. (2007). Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nat Protoc* 2(5), 1254-1262. doi: 10.1038/nprot.2007.158.
- Francis, V.I., Stevenson, E.C., and Porter, S.L. (2017). Two-component systems required for virulence in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 364(11). doi: 10.1093/femsle/fnx104.
- Fuchs, E.L., Brutinel, E.D., Jones, A.K., Fulcher, N.B., Urbanowski, M.L., Yahr, T.L., et al. (2010). The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. *J Bacteriol* 192(14), 3553-3564. doi: 10.1128/JB.00363-10.
- Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* 50, 727-751. doi: 10.1146/annurev.micro.50.1.727.
- Furstenberg-Hagg, J., Zagrobelny, M., and Bak, S. (2013). Plant defense against insect herbivores. *Int J Mol Sci* 14(5), 10242-10297. doi: 10.3390/ijms140510242.
- Gaines, J.M., Carty, N.L., Tiburzi, F., Davinic, M., Visca, P., Colmer-Hamood, J.A., et al. (2007). Regulation of the *Pseudomonas aeruginosa* *toxA*, *regA* and *ptxR* genes by the iron-starvation sigma factor PvdS under reduced levels of oxygen. *Microbiology (Reading)* 153(Pt 12), 4219-4233. doi: 10.1099/mic.0.2007/011338-0.
- Galan-Vasquez, E., Luna, B., and Martinez-Antonio, A. (2011). The Regulatory Network of *Pseudomonas aeruginosa*. *Microb Inform Exp* 1(1), 3. doi: 10.1186/2042-5783-1-3.
- Gallagher, L.A., and Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* 183(21), 6207-6214. doi: 10.1128/JB.183.21.6207-6214.2001.
- Gallagher, L.A., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C. (2002). Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* 184(23), 6472-6480. doi: 10.1128/jb.184.23.6472-6480.2002.
- Garcia-Contreras, R., Perez-Eretza, B., Jasso-Chavez, R., Lira-Silva, E., Roldan-Sanchez, J.A., Gonzalez-Valdez, A., et al. (2015). High variability in quorum quenching and growth inhibition by furanone C-30 in *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients. *Pathog Dis* 73(6), ftv040. doi: 10.1093/femspd/ftv040.
- Gellatly, S.L., and Hancock, R.E. (2013). *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathog Dis* 67(3), 159-173. doi: 10.1111/2049-632X.12033.
- Gicquel, G., Bouffartigues, E., Bains, M., Oxaran, V., Rosay, T., Lesouhaitier, O., et al. (2013). The extra-cytoplasmic function sigma factor sigX modulates biofilm and virulence-related properties in *Pseudomonas aeruginosa*. *PLoS One* 8(11), e80407. doi: 10.1371/journal.pone.0080407.
- Gokalsin, B., Aksoydan, B., Erman, B., and Sesal, N.C. (2017). Reducing Virulence and Biofilm of *Pseudomonas aeruginosa* by Potential Quorum Sensing Inhibitor Carotenoid: Zeaxanthin. *Microb Ecol* 74(2), 466-473. doi: 10.1007/s00248-017-0949-3.

- Gooderham, W.J., and Hancock, R.E. (2009). Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 33(2), 279-294. doi: 10.1111/j.1574-6976.2008.00135.x.
- Goodman, A.L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R.S., and Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 7(5), 745-754. doi: 10.1016/j.devcel.2004.08.020.
- Grabiner, M.A., Fu, Z., Wu, T., Barry, K.C., Schwarzer, C., and Machen, T.E. (2014). *Pseudomonas aeruginosa* quorum-sensing molecule homoserine lactone modulates inflammatory signaling through PERK and eIF2 α . *J Immunol* 193(3), 1459-1467. doi: 10.4049/jimmunol.1303437.
- Grandclement, C., Tannieres, M., Morera, S., Dessaux, Y., and Faure, D. (2016). Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev* 40(1), 86-116. doi: 10.1093/femsre/fuv038.
- Guerinot, M.L. (1994). Microbial iron transport. *Annu Rev Microbiol* 48, 743-772. doi: 10.1146/annurev.mi.48.100194.003523.
- Guerinot, M.L., and Yi, Y. (1994). Iron: Nutritious, Noxious, and Not Readily Available. *Plant Physiol* 104(3), 815-820. doi: 10.1104/pp.104.3.815.
- Ha, D.G., and O'Toole, G.A. (2015). c-di-GMP and its Effects on Biofilm Formation and Dispersion: a *Pseudomonas aeruginosa* Review. *Microbiol Spectr* 3(2), MB-0003-2014. doi: 10.1128/microbiolspec.MB-0003-2014.
- Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A.J., Forbes, A., Perkins, A.V., et al. (2016). Cellular Effects of Pyocyanin, a Secreted Virulence Factor of *Pseudomonas aeruginosa*. *Toxins (Basel)* 8(8). doi: 10.3390/toxins8080236.
- Hancock, R.E. (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin Infect Dis* 27 Suppl 1, S93-99. doi: 10.1086/514909.
- Hanzelka, B.L., and Greenberg, E.P. (1996). Quorum sensing in *Vibrio fischeri*: evidence that S-adenosylmethionine is the amino acid substrate for autoinducer synthesis. *J Bacteriol* 178(17), 5291-5294. doi: 10.1128/jb.178.17.5291-5294.1996.
- Hauser, A.R. (2009). The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* 7(9), 654-665. doi: 10.1038/nrmicro2199.
- Haussler, S. (2004). Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ Microbiol* 6(6), 546-551. doi: 10.1111/j.1462-2920.2004.00618.x.
- Heck, L.W., Alarcon, P.G., Kulhavy, R.M., Morihara, K., Russell, M.W., and Mestecky, J.F. (1990). Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. *J Immunol* 144(6), 2253-2257.
- Hemaiswarya, S., Kruthiventi, A.K., and Doble, M. (2008). Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine* 15(8), 639-652. doi: 10.1016/j.phymed.2008.06.008.
- Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., et al. (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* 22(15), 3803-3815. doi: 10.1093/emboj/cdg366.
- Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., et al. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 186(10), 2936-2945. doi: 10.1128/jb.186.10.2936-2945.2004.

- Hirakata, Y., Kondo, A., Hoshino, K., Yano, H., Arai, K., Hirotsu, A., et al. (2009). Efflux pump inhibitors reduce the invasiveness of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* 34(4), 343-346. doi: 10.1016/j.ijantimicag.2009.06.007.
- Hirakawa, H., and Tomita, H. (2013). Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance. *Front Microbiol* 4, 114. doi: 10.3389/fmicb.2013.00114.
- Hoang, S., Georget, A., Asselineau, J., Venier, A.G., Leroyer, C., Rogues, A.M., et al. (2018). Risk factors for colonization and infection by *Pseudomonas aeruginosa* in patients hospitalized in intensive care units in France. *PLoS One* 13(3), e0193300. doi: 10.1371/journal.pone.0193300.
- Huang, J.J., Petersen, A., Whiteley, M., and Leadbetter, J.R. (2006). Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol* 72(2), 1190-1197. doi: 10.1128/AEM.72.2.1190-1197.2006.
- Irie, Y., Starkey, M., Edwards, A.N., Wozniak, D.J., Romeo, T., and Parsek, M.R. (2010). *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol* 78(1), 158-172. doi: 10.1111/j.1365-2958.2010.07320.x.
- Jakobsen, T.H., Warming, A.N., Vejborg, R.M., Moscoso, J.A., Stegger, M., Lorenzen, F., et al. (2017). A broad range quorum sensing inhibitor working through sRNA inhibition. *Sci Rep* 7(1), 9857. doi: 10.1038/s41598-017-09886-8.
- Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M.A., et al. (2018). Bacterial biofilm and associated infections. *J Chin Med Assoc* 81(1), 7-11. doi: 10.1016/j.jcma.2017.07.012.
- Jefferson, K.K., Goldmann, D.A., and Pier, G.B. (2005). Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother* 49(6), 2467-2473. doi: 10.1128/AAC.49.6.2467-2473.2005.
- Jimenez, P.N., Koch, G., Thompson, J.A., Xavier, K.B., Cool, R.H., and Quax, W.J. (2012). The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 76(1), 46-65. doi: 10.1128/MMBR.05007-11.
- Juhas, M., Wiehlmann, L., Huber, B., Jordan, D., Lauber, J., Salunkhe, P., et al. (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology (Reading)* 150(Pt 4), 831-841. doi: 10.1099/mic.0.26906-0.
- Kanack, K.J., Runyen-Janecky, L.J., Ferrell, E.P., Suh, S.J., and West, S.E.H. (2006). Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. *Microbiology* 152(Pt 12), 3485-3496. doi: 10.1099/mic.0.29008-0.
- Kanno, E., Kawakami, K., Miyairi, S., Tanno, H., Otomaru, H., Hatanaka, A., et al. (2013). Neutrophil-derived tumor necrosis factor-alpha contributes to acute wound healing promoted by N-(3-oxododecanoyl)-L-homoserine lactone from *Pseudomonas aeruginosa*. *J Dermatol Sci* 70(2), 130-138. doi: 10.1016/j.jdermsci.2013.01.004.
- Kanno, E., Kawakami, K., Miyairi, S., Tanno, H., Suzuki, A., Kamimatsuno, R., et al. (2016). Promotion of acute-phase skin wound healing by *Pseudomonas aeruginosa* C4 -HSL. *Int Wound J* 13(6), 1325-1335. doi: 10.1111/iwj.12523.
- Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., et al. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* 188(16), 6026-6033. doi: 10.1128/JB.00409-06.

- Khajanchi, B.K., Kirtley, M.L., Brackman, S.M., and Chopra, A.K. (2011). Immunomodulatory and protective roles of quorum-sensing signaling molecules N-acyl homoserine lactones during infection of mice with *Aeromonas hydrophila*. *Infect Immun* 79(7), 2646-2657. doi: 10.1128/IAI.00096-11.
- Kim, H.S., and Park, H.D. (2013). Ginger extract inhibits biofilm formation by *Pseudomonas aeruginosa* PA14. *PLoS One* 8(9), e76106. doi: 10.1371/journal.pone.0076106.
- Kim, K., Kim, Y.U., Koh, B.H., Hwang, S.S., Kim, S.H., Lepine, F., et al. (2010). HHQ and PQS, two *Pseudomonas aeruginosa* quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway. *Immunology* 129(4), 578-588. doi: 10.1111/j.1365-2567.2009.03160.x.
- Kipnis, E., Sawa, T., and Wiener-Kronish, J. (2006). Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. *Med Mal Infect* 36(2), 78-91. doi: 10.1016/j.medmal.2005.10.007.
- Kohler, T., Curty, L.K., Barja, F., van Delden, C., and Pechere, J.C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* 182(21), 5990-5996. doi: 10.1128/jb.182.21.5990-5996.2000.
- Krzyzek, P. (2019). Challenges and Limitations of Anti-quorum Sensing Therapies. *Front Microbiol* 10, 2473. doi: 10.3389/fmicb.2019.02473.
- Kuang, Z., Hao, Y., Hwang, S., Zhang, S., Kim, E., Akinbi, H.T., et al. (2011). The *Pseudomonas aeruginosa* flagellum confers resistance to pulmonary surfactant protein-A by impacting the production of exoproteases through quorum-sensing. *Mol Microbiol* 79(5), 1220-1235. doi: 10.1111/j.1365-2958.2010.07516.x.
- Kumar, V.P., Chauhan, N.S., Padh, H., and Rajani, M. (2006). Search for antibacterial and antifungal agents from selected Indian medicinal plants. *J Ethnopharmacol* 107(2), 182-188. doi: 10.1016/j.jep.2006.03.013.
- LaSarre, B., and Federle, M.J. (2013). Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev* 77(1), 73-111. doi: 10.1128/MMBR.00046-12.
- Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* 21(6), 1137-1146. doi: 10.1046/j.1365-2958.1996.00063.x.
- Lau, G.W., Hassett, D.J., Ran, H., and Kong, F. (2004). The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* 10(12), 599-606. doi: 10.1016/j.molmed.2004.10.002.
- Laughlin, R.S., Musch, M.W., Hollbrook, C.J., Rocha, F.M., Chang, E.B., and Alverdy, J.C. (2000). The key role of *Pseudomonas aeruginosa* PA-I lectin on experimental gut-derived sepsis. *Ann Surg* 232(1), 133-142. doi: 10.1097/00000658-200007000-00019.
- Lebeaux, D., Ghigo, J.M., and Beloin, C. (2014). Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol Rev* 78(3), 510-543. doi: 10.1128/MMBR.00013-14.
- Lee, B., and Boucher, H.W. (2015). Targeting antimicrobial-resistant bacterial respiratory tract pathogens: it is time to 'get smart'. *Curr Opin Pulm Med* 21(3), 293-303. doi: 10.1097/MCP.0000000000000157.
- Leisinger, T., and Margraff, R. (1979). Secondary metabolites of the fluorescent pseudomonads. *Microbiol Rev* 43(3), 422-442.
- Lepine, F., Deziel, E., Milot, S., and Rahme, L.G. (2003). A stable isotope dilution assay for the quantification of the *Pseudomonas* quinolone signal in *Pseudomonas aeruginosa* cultures. *Biochim Biophys Acta* 1622(1), 36-41. doi: 10.1016/s0304-4165(03)00103-x.

- Lepine, F., Milot, S., Deziel, E., He, J., and Rahme, L.G. (2004). Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*. *J Am Soc Mass Spectrom* 15(6), 862-869. doi: 10.1016/j.jasms.2004.02.012.
- Lerouge, I., and Vanderleyden, J. (2002). O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiol Rev* 26(1), 17-47. doi: 10.1111/j.1574-6976.2002.tb00597.x.
- Lewis, K., and Ausubel, F.M. (2006). Prospects for plant-derived antibacterials. *Nat Biotechnol* 24(12), 1504-1507. doi: 10.1038/nbt1206-1504.
- Liang-Takasaki, C.J., Grossman, N., and Leive, L. (1983). *Salmonellae* activate complement differentially via the alternative pathway depending on the structure of their lipopolysaccharide O-antigen. *J Immunol* 130(4), 1867-1870.
- Liang-Takasaki, C.J., Makela, P.H., and Leive, L. (1982). Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. *J Immunol* 128(3), 1229-1235.
- Lin, J., Cheng, J., Wang, Y., and Shen, X. (2018). The *Pseudomonas* Quinolone Signal (PQS): Not Just for Quorum Sensing Anymore. *Front Cell Infect Microbiol* 8, 230. doi: 10.3389/fcimb.2018.00230.
- Losa, D., Kohler, T., Bacchetta, M., Saab, J.B., Frieden, M., van Delden, C., et al. (2015). Airway Epithelial Cell Integrity Protects from Cytotoxicity of *Pseudomonas aeruginosa* Quorum-Sensing Signals. *Am J Respir Cell Mol Biol* 53(2), 265-275. doi: 10.1165/rcmb.2014-0405OC.
- Lujan, A.M., Moyano, A.J., Segura, I., Argarana, C.E., and Smania, A.M. (2007). Quorum-sensing-deficient (*lasR*) mutants emerge at high frequency from a *Pseudomonas aeruginosa* *mutS* strain. *Microbiology (Reading)* 153(Pt 1), 225-237. doi: 10.1099/mic.0.29021-0.
- Lyczak, J.B., Cannon, C.L., and Pier, G.B. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* 2(9), 1051-1060. doi: 10.1016/s1286-4579(00)01259-4.
- Lyczak, J.B., Cannon, C.L., and Pier, G.B. (2002). Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 15(2), 194-222. doi: 10.1128/cmr.15.2.194-222.2002.
- Ma, L., Jackson, K.D., Landry, R.M., Parsek, M.R., and Wozniak, D.J. (2006). Analysis of *Pseudomonas aeruginosa* conditional *psl* variants reveals roles for the *psl* polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* 188(23), 8213-8221. doi: 10.1128/JB.01202-06.
- Maeda, T., Garcia-Contreras, R., Pu, M., Sheng, L., Garcia, L.R., Tomas, M., et al. (2012). Quorum quenching quandary: resistance to antivirulence compounds. *ISME J* 6(3), 493-501. doi: 10.1038/ismej.2011.122.
- Mahajan-Miklos, S., Tan, M.W., Rahme, L.G., and Ausubel, F.M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* 96(1), 47-56. doi: 10.1016/s0092-8674(00)80958-7.
- Malloy, J.L., Veldhuizen, R.A., Thibodeaux, B.A., O'Callaghan, R.J., and Wright, J.R. (2005). *Pseudomonas aeruginosa* protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions. *Am J Physiol Lung Cell Mol Physiol* 288(2), L409-418. doi: 10.1152/ajplung.00322.2004.
- Mann, E.E., and Wozniak, D.J. (2012). *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiol Rev* 36(4), 893-916. doi: 10.1111/j.1574-6976.2011.00322.x.

- Mariencheck, W.I., Alcorn, J.F., Palmer, S.M., and Wright, J.R. (2003). *Pseudomonas aeruginosa* elastase degrades surfactant proteins A and D. *Am J Respir Cell Mol Biol* 28(4), 528-537. doi: 10.1165/rcmb.2002-0141OC.
- Mashburn, L.M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437(7057), 422-425. doi: 10.1038/nature03925.
- Matsumoto, K. (2004). Role of bacterial proteases in pseudomonal and serratial keratitis. *Biol Chem* 385(11), 1007-1016. doi: 10.1515/BC.2004.131.
- Maura, D., Hazan, R., Kitao, T., Ballok, A.E., and Rahme, L.G. (2016). Evidence for Direct Control of Virulence and Defense Gene Circuits by the *Pseudomonas aeruginosa* Quorum Sensing Regulator, MvfR. *Sci Rep* 6, 34083. doi: 10.1038/srep34083.
- Mavrodi, D.V., Bonsall, R.F., Delaney, S.M., Soule, M.J., Phillips, G., and Thomashow, L.S. (2001). Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 183(21), 6454-6465. doi: 10.1128/JB.183.21.6454-6465.2001.
- McClellan, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., et al. (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology (Reading)* 143 (Pt 12), 3703-3711. doi: 10.1099/00221287-143-12-3703.
- McGrath, S., Wade, D.S., and Pesci, E.C. (2004). Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). *FEMS Microbiol Lett* 230(1), 27-34. doi: 10.1016/S0378-1097(03)00849-8.
- McKnight, S.L., Iglewski, B.H., and Pesci, E.C. (2000). The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 182(10), 2702-2708. doi: 10.1128/jb.182.10.2702-2708.2000.
- Meyer, J.M., Neely, A., Stintzi, A., Georges, C., and Holder, I.A. (1996). Pyoverdinin is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun* 64(2), 518-523. doi: 10.1128/IAI.64.2.518-523.1996.
- Michel, L., Gonzalez, N., Jagdeep, S., Nguyen-Ngoc, T., and Reimmann, C. (2005). PchR-box recognition by the AraC-type regulator PchR of *Pseudomonas aeruginosa* requires the siderophore pyochelin as an effector. *Mol Microbiol* 58(2), 495-509. doi: 10.1111/j.1365-2958.2005.04837.x.
- Miyairi, S., Tateda, K., Fuse, E.T., Ueda, C., Saito, H., Takabatake, T., et al. (2006). Immunization with 3-oxododecanoyl-L-homoserine lactone-protein conjugate protects mice from lethal *Pseudomonas aeruginosa* lung infection. *J Med Microbiol* 55(Pt 10), 1381-1387. doi: 10.1099/jmm.0.46658-0.
- Moradali, M.F., Ghods, S., and Rehm, B.H. (2017). *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* 7, 39. doi: 10.3389/fcimb.2017.00039.
- Muhlen, S., and Dersch, P. (2016). Anti-virulence Strategies to Target Bacterial Infections. *Curr Top Microbiol Immunol* 398, 147-183. doi: 10.1007/82_2015_490.
- Mulcahy, H., O'Callaghan, J., O'Grady, E.P., Adams, C., and O'Gara, F. (2006). The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect Immun* 74(5), 3012-3015. doi: 10.1128/IAI.74.5.3012-3015.2006.
- Mulcahy, L.R., Isabella, V.M., and Lewis, K. (2014). *Pseudomonas aeruginosa* biofilms in disease. *Microb Ecol* 68(1), 1-12. doi: 10.1007/s00248-013-0297-x.

- Nealson, K.H., Platt, T., and Hastings, J.W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* 104(1), 313-322. doi: 10.1128/JB.104.1.313-322.1970.
- Ng, W.L., and Bassler, B.L. (2009). Bacterial quorum-sensing network architectures. *Annu Rev Genet* 43, 197-222. doi: 10.1146/annurev-genet-102108-134304.
- Nomura, K., Obata, K., Keira, T., Miyata, R., Hirakawa, S., Takano, K., et al. (2014). *Pseudomonas aeruginosa* elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells. *Respir Res* 15, 21. doi: 10.1186/1465-9921-15-21.
- O'Toole, G.A., and Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 30(2), 295-304. doi: 10.1046/j.1365-2958.1998.01062.x.
- Oliva, G., Sahr, T., and Buchrieser, C. (2015). Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence. *FEMS Microbiol Rev* 39(3), 331-349. doi: 10.1093/femsre/fuv022.
- Oliveira, V.C., Bim, F.L., Monteiro, R.M., Macedo, A.P., Santos, E.S., Silva-Lovato, C.H., et al. (2020). Identification and Characterization of New Bacteriophages to Control Multidrug-Resistant *Pseudomonas aeruginosa* Biofilm on Endotracheal Tubes. *Front Microbiol* 11, 580779. doi: 10.3389/fmicb.2020.580779.
- Ortiz, S., Lecso-Bornet, M., Bonnal, C., Houze, S., Michel, S., Grougnet, R., et al. (2019). Bioguided identification of triterpenoids and neolignans as bioactive compounds from anti-infectious medicinal plants of the Taira Atacama's community (Calama, Chile). *J Ethnopharmacol* 231, 217-229.
- Otero-Asman, J.R., Garcia-Garcia, A.I., Civantos, C., Quesada, J.M., and Llamas, M.A. (2019a). *Pseudomonas aeruginosa* possesses three distinct systems for sensing and using the host molecule haem. *Environ Microbiol* 21(12), 4629-4647. doi: 10.1111/1462-2920.14773.
- Otero-Asman, J.R., Wettstadt, S., Bernal, P., and Llamas, M.A. (2019b). Diversity of extracytoplasmic function sigma (sigma(ECF)) factor-dependent signaling in *Pseudomonas*. *Mol Microbiol* 112(2), 356-373. doi: 10.1111/mmi.14331.
- Pachori, P., Goyalwal, R., and Gandhi, P. (2019). Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. *Genes Dis* 6(2), 109-119. doi: 10.1016/j.gendis.2019.04.001.
- Pamp, S.J., and Tolker-Nielsen, T. (2007). Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol* 189(6), 2531-2539. doi: 10.1128/JB.01515-06.
- Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan, J.E., Jr., and Greenberg, E.P. (1999). Acyl homoserine-lactone quorum-sensing signal generation. *Proc Natl Acad Sci U S A* 96(8), 4360-4365. doi: 10.1073/pnas.96.8.4360.
- Passos da Silva, D., Matwichuk, M.L., Townsend, D.O., Reichhardt, C., Lamba, D., Wozniak, D.J., et al. (2019). The *Pseudomonas aeruginosa* lectin LecB binds to the exopolysaccharide Psl and stabilizes the biofilm matrix. *Nat Commun* 10(1), 2183. doi: 10.1038/s41467-019-10201-4.
- Paul, M., and Leibovici, L. (2009). Combination antimicrobial treatment versus monotherapy: the contribution of meta-analyses. *Infect Dis Clin North Am* 23(2), 277-293. doi: 10.1016/j.idc.2009.01.004.
- Pearson, J.P., Van Delden, C., and Iglewski, B.H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* 181(4), 1203-1210. doi: 10.1128/JB.181.4.1203-1210.1999.

- Pedersen, B.K., Kharazmi, A., Theander, T.G., Odum, N., Andersen, V., and Bendtzen, K. (1987). Selective modulation of the CD4 molecular complex by *Pseudomonas aeruginosa* alkaline protease and elastase. *Scand J Immunol* 26(1), 91-94. doi: 10.1111/j.1365-3083.1987.tb02239.x.
- Pedersen, S.S., Hoiby, N., Espersen, F., and Koch, C. (1992). Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 47(1), 6-13. doi: 10.1136/thx.47.1.6.
- Pena, R.T., Blasco, L., Ambroa, A., Gonzalez-Pedrajo, B., Fernandez-Garcia, L., Lopez, M., et al. (2019). Relationship Between Quorum Sensing and Secretion Systems. *Front Microbiol* 10, 1100. doi: 10.3389/fmicb.2019.01100.
- Perumal Samy, R., and Gopalakrishnakone, P. (2010). Therapeutic Potential of Plants as Antimicrobials for Drug Discovery. *Evid Based Complement Alternat Med* 7(3), 283-294. doi: 10.1093/ecam/nen036.
- Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997). Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179(10), 3127-3132. doi: 10.1128/jb.179.10.3127-3132.1997.
- Pirnay, J.P. (2020). Phage Therapy in the Year 2035. *Front Microbiol* 11, 1171. doi: 10.3389/fmicb.2020.01171.
- Pirnay, J.P., Bilocq, F., Pot, B., Cornelis, P., Zizi, M., Van Eldere, J., et al. (2009). *Pseudomonas aeruginosa* population structure revisited. *PLoS One* 4(11), e7740. doi: 10.1371/journal.pone.0007740.
- Pletzer, D., Blimkie, T.M., Wolfmeier, H., Li, Y., Baghela, A., Lee, A.H.Y., et al. (2020). The Stringent Stress Response Controls Proteases and Global Regulators under Optimal Growth Conditions in *Pseudomonas aeruginosa*. *mSystems* 5(4). doi: 10.1128/mSystems.00495-20.
- Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* 2, 65. doi: 10.3389/fmicb.2011.00065.
- Popat, R., Cruz, S.A., and Diggle, S.P. (2008). The social behaviours of bacterial pathogens. *Br Med Bull* 87, 63-75. doi: 10.1093/bmb/ldn030.
- Price-Whelan, A., Dietrich, L.E., and Newman, D.K. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* 2(2), 71-78. doi: 10.1038/nchembio764.
- Pustelny, C., Albers, A., Buldt-Karentzopoulos, K., Parschat, K., Chhabra, S.R., Camara, M., et al. (2009). Dioxygenase-mediated quenching of quinolone-dependent quorum sensing in *Pseudomonas aeruginosa*. *Chem Biol* 16(12), 1259-1267. doi: 10.1016/j.chembiol.2009.11.013.
- Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V., and Leoni, L. (2006). The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the *lasI* promoter. *J Bacteriol* 188(2), 815-819. doi: 10.1128/JB.188.2.815-819.2006.
- Rampioni, G., Falcone, M., Heeb, S., Frangipani, E., Fletcher, M.P., Dubern, J.F., et al. (2016). Unravelling the Genome-Wide Contributions of Specific 2-Alkyl-4-Quinolones and PqsE to Quorum Sensing in *Pseudomonas aeruginosa*. *PLoS Pathog* 12(11), e1006029. doi: 10.1371/journal.ppat.1006029.
- Rampioni, G., Pillai, C.R., Longo, F., Bondi, R., Baldelli, V., Messina, M., et al. (2017). Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence. *Sci Rep* 7(1), 11392. doi: 10.1038/s41598-017-11892-9.
- Ramsey, D.M., and Wozniak, D.J. (2005). Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol Microbiol* 56(2), 309-322. doi: 10.1111/j.1365-2958.2005.04552.x.

- Rasamiravaka, T., Labtani, Q., Duez, P., and El Jaziri, M. (2015a). The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. *Biomed Res Int* 2015, 759348. doi: 10.1155/2015/759348.
- Rasamiravaka, T., Vandeputte, O.M., Pottier, L., Huet, J., Rabemanantsoa, C., Kiendrebeogo, M., et al. (2015b). *Pseudomonas aeruginosa* Biofilm Formation and Persistence, along with the Production of Quorum Sensing-Dependent Virulence Factors, Are Disrupted by a Triterpenoid Coumarate Ester Isolated from *Dalbergia trichocarpa*, a Tropical Legume. *PLoS One* 10(7), e0132791. doi: 10.1371/journal.pone.0132791.
- Rasko, D.A., Moreira, C.G., Li de, R., Reading, N.C., Ritchie, J.M., Waldor, M.K., et al. (2008). Targeting QseC signaling and virulence for antibiotic development. *Science* 321(5892), 1078-1080. doi: 10.1126/science.1160354.
- Rasko, D.A., and Sperandio, V. (2010). Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9(2), 117-128. doi: 10.1038/nrd3013.
- Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kote, M., et al. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *J Bacteriol* 187(5), 1799-1814. doi: 10.1128/JB.187.5.1799-1814.2005.
- Rasmussen, T.B., and Givskov, M. (2006). Quorum-sensing inhibitors as anti-pathogenic drugs. *Int J Med Microbiol* 296(2-3), 149-161. doi: 10.1016/j.ijmm.2006.02.005.
- Ratledge, C., and Dover, L.G. (2000). Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* 54, 881-941. doi: 10.1146/annurev.micro.54.1.881.
- Rauf, A., Patel, S., Uddin, G., Siddiqui, B.S., Ahmad, B., Muhammad, N., et al. (2017). Phytochemical, ethnomedicinal uses and pharmacological profile of genus *Pistacia*. *Biomed Pharmacother* 86, 393-404. doi: 10.1016/j.biopha.2016.12.017.
- Reichhardt, C., and Parsek, M.R. (2019). Confocal Laser Scanning Microscopy for Analysis of *Pseudomonas aeruginosa* Biofilm Architecture and Matrix Localization. *Front Microbiol* 10, 677. doi: 10.3389/fmicb.2019.00677.
- Reimann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., et al. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* 24(2), 309-319. doi: 10.1046/j.1365-2958.1997.3291701.x.
- Rutherford, S.T., and Bassler, B.L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med* 2(11). doi: 10.1101/cshperspect.a012427.
- Ryder, C., Byrd, M., and Wozniak, D.J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol* 10(6), 644-648. doi: 10.1016/j.mib.2007.09.010.
- Sana, T.G., Berni, B., and Bleves, S. (2016). The T6SSs of *Pseudomonas aeruginosa* Strain PAO1 and Their Effectors: Beyond Bacterial-Cell Targeting. *Front Cell Infect Microbiol* 6, 61. doi: 10.3389/fcimb.2016.00061.
- Sandasi, M., Leonard, C.M., and Viljoen, A.M. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Lett Appl Microbiol* 50(1), 30-35. doi: 10.1111/j.1472-765X.2009.02747.x.
- Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., and Davies, D.G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184(4), 1140-1154. doi: 10.1128/jb.184.4.1140-1154.2002.

- Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A., Davies, D.G., and Gilbert, P. (2004). Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* 186(21), 7312-7326. doi: 10.1128/JB.186.21.7312-7326.2004.
- Schalk, I.J., Abdallah, M.A., and Pattus, F. (2002). A new mechanism for membrane iron transport in *Pseudomonas aeruginosa*. *Biochem Soc Trans* 30(4), 702-705. doi: 10.1042/bst0300702.
- Schuster, M., and Greenberg, E.P. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* 296(2-3), 73-81. doi: 10.1016/j.ijmm.2006.01.036.
- Schuster, M., Hawkins, A.C., Harwood, C.S., and Greenberg, E.P. (2004). The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 51(4), 973-985. doi: 10.1046/j.1365-2958.2003.03886.x.
- Schutz, C., and Empting, M. (2018). Targeting the *Pseudomonas* quinolone signal quorum sensing system for the discovery of novel anti-infective pathoblockers. *Beilstein J Org Chem* 14, 2627-2645. doi: 10.3762/bjoc.14.241.
- Schweizer, H.P. (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet Mol Res* 2(1), 48-62.
- Seet, Q., and Zhang, L.H. (2011). Anti-activator QslA defines the quorum sensing threshold and response in *Pseudomonas aeruginosa*. *Mol Microbiol* 80(4), 951-965. doi: 10.1111/j.1365-2958.2011.07622.x.
- Selezska, K., Kazmierczak, M., Musken, M., Garbe, J., Schobert, M., Haussler, S., et al. (2012). *Pseudomonas aeruginosa* population structure revisited under environmental focus: impact of water quality and phage pressure. *Environ Microbiol* 14(8), 1952-1967. doi: 10.1111/j.1462-2920.2012.02719.x.
- Silva, L.N., Zimmer, K.R., Macedo, A.J., and Trentin, D.S. (2016). Plant Natural Products Targeting Bacterial Virulence Factors. *Chem Rev* 116(16), 9162-9236. doi: 10.1021/acs.chemrev.6b00184.
- Simm, R., Morr, M., Kader, A., Nimtz, M., and Romling, U. (2004). GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* 53(4), 1123-1134. doi: 10.1111/j.1365-2958.2004.04206.x.
- Sio, C.F., Otten, L.G., Cool, R.H., Diggle, S.P., Braun, P.G., Bos, R., et al. (2006). Quorum quenching by an N-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infect Immun* 74(3), 1673-1682. doi: 10.1128/IAI.74.3.1673-1682.2006.
- Sitnikov, D.M., Schineller, J.B., and Baldwin, T.O. (1995). Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. *Mol Microbiol* 17(5), 801-812. doi: 10.1111/j.1365-2958.1995.mmi_17050801.x.
- Smith, R.S., and Iglewski, B.H. (2003). *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* 6(1), 56-60. doi: 10.1016/s1369-5274(03)00008-0.
- Smith, W.D., Bardin, E., Cameron, L., Edmondson, C.L., Farrant, K.V., Martin, I., et al. (2017). Current and future therapies for *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *FEMS Microbiol Lett* 364(14). doi: 10.1093/femsle/fnx121.
- Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A.S., Backofen, R., et al. (2011). The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Mol Microbiol* 80(4), 868-885. doi: 10.1111/j.1365-2958.2011.07620.x.
- Starkey, M., Hickman, J.H., Ma, L., Zhang, N., De Long, S., Hinz, A., et al. (2009). *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J Bacteriol* 191(11), 3492-3503. doi: 10.1128/JB.00119-09.

- Stoltz, D.A., Ozer, E.A., Taft, P.J., Barry, M., Liu, L., Kiss, P.J., et al. (2008). *Drosophila* are protected from *Pseudomonas aeruginosa* lethality by transgenic expression of paraoxonase-1. *J Clin Invest* 118(9), 3123-3131. doi: 10.1172/JCI35147.
- Stoodley, P., Cargo, R., Rupp, C.J., Wilson, S., and Klapper, I. (2002). Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J Ind Microbiol Biotechnol* 29(6), 361-367. doi: 10.1038/sj.jim.7000282.
- Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., et al. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406(6799), 959-964. doi: 10.1038/35023079.
- Tahrioui, A., Ortiz, S., Azuama, O.C., Bouffartigues, E., Benalia, N., Tortuel, D., et al. (2020). Membrane-Interactive Compounds From *Pistacia lentiscus* L. Thwart *Pseudomonas aeruginosa* Virulence. *Front Microbiol* 11, 1068. doi: 10.3389/fmicb.2020.01068.
- Tan, S.Y., Liu, Y., Chua, S.L., Vejborg, R.M., Jakobsen, T.H., Chew, S.C., et al. (2014). Comparative systems biology analysis to study the mode of action of the isothiocyanate compound Iberin on *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 58(11), 6648-6659. doi: 10.1128/AAC.02620-13.
- Teplitski, M., Robinson, J.B., and Bauer, W.D. (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol Plant Microbe Interact* 13(6), 637-648. doi: 10.1094/MPMI.2000.13.6.637.
- Thanassi, D.G., Bliska, J.B., and Christie, P.J. (2012). Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function. *FEMS Microbiol Rev* 36(6), 1046-1082. doi: 10.1111/j.1574-6976.2012.00342.x.
- Theander, T.G., Kharazmi, A., Pedersen, B.K., Christensen, L.D., Tvede, N., Poulsen, L.K., et al. (1988). Inhibition of human lymphocyte proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases. *Infect Immun* 56(7), 1673-1677. doi: 10.1128/IAI.56.7.1673-1677.1988.
- Thibodeau, P.H., and Butterworth, M.B. (2013). Proteases, cystic fibrosis and the epithelial sodium channel (ENaC). *Cell Tissue Res* 351(2), 309-323. doi: 10.1007/s00441-012-1439-z.
- Thompson, C.M., and Malone, J.G. (2020). Nucleotide second messengers in bacterial decision making. *Curr Opin Microbiol* 55, 34-39. doi: 10.1016/j.mib.2020.02.006.
- Torres, R., Faini, F., Delle Monache, F., and Delle Monache, G. (2004). Two new O-geranyl coumarins from the resinous exudate of *Haplopappus multifolius*. *Fitoterapia* 75(1), 5-8. doi: 10.1016/j.fitote.2003.06.003.
- Torres, R., Faini, F., Modak, B., Urbina, F., Labbe, C., and Guerrero, J. (2006). Antioxidant activity of coumarins and flavonols from the resinous exudate of *Haplopappus multifolius*. *Phytochemistry* 67(10), 984-987. doi: 10.1016/j.phytochem.2006.03.016.
- Turkina, M.V., and Vikstrom, E. (2019). Bacteria-Host Crosstalk: Sensing of the Quorum in the Context of *Pseudomonas aeruginosa* Infections. *J Innate Immun* 11(3), 263-279. doi: 10.1159/000494069.
- Vakulskas, C.A., Potts, A.H., Babitzke, P., Ahmer, B.M., and Romeo, T. (2015). Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol Mol Biol Rev* 79(2), 193-224. doi: 10.1128/MMBR.00052-14.
- Valentini, M., and Filloux, A. (2016). Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *J Biol Chem* 291(24), 12547-12555. doi: 10.1074/jbc.R115.711507.

- Valentini, M., Gonzalez, D., Mavridou, D.A., and Filloux, A. (2018). Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*. *Curr Opin Microbiol* 41, 15-20. doi: 10.1016/j.mib.2017.11.006.
- Vallet, I., Olson, J.W., Lory, S., Lazdunski, A., and Filloux, A. (2001). The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. *Proc Natl Acad Sci U S A* 98(12), 6911-6916. doi: 10.1073/pnas.111551898.
- van Delden, C., Comte, R., and Bally, A.M. (2001). Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 183(18), 5376-5384. doi: 10.1128/jb.183.18.5376-5384.2001.
- Van Delden, C., and Iglewski, B.H. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* 4(4), 551-560. doi: 10.3201/eid0404.980405.
- VanEtten, H.D., Mansfield, J.W., Bailey, J.A., and Farmer, E.E. (1994). Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins". *Plant Cell* 6(9), 1191-1192. doi: 10.1105/tpc.6.9.1191.
- Vasavi, H.S., Arun, A.B., and Rekha, P.D. (2016). Anti-quorum sensing activity of flavonoid-rich fraction from *Centella asiatica* L. against *Pseudomonas aeruginosa* PAO1. *J Microbiol Immunol Infect* 49(1), 8-15. doi: 10.1016/j.jmii.2014.03.012.
- Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., et al. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* 103(1), 171-176. doi: 10.1073/pnas.0507407103.
- Vilaplana, L., and Marco, M.P. (2020). Phenazines as potential biomarkers of *Pseudomonas aeruginosa* infections: synthesis regulation, pathogenesis and analytical methods for their detection. *Anal Bioanal Chem* 412(24), 5897-5912. doi: 10.1007/s00216-020-02696-4.
- Visca, P., Colotti, G., Serino, L., Verzili, D., Orsi, N., and Chiancone, E. (1992). Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. *Appl Environ Microbiol* 58(9), 2886-2893. doi: 10.1128/AEM.58.9.2886-2893.1992.
- Visca, P., and Imperi, F. (2018). An essential transcriptional regulator: the case of *Pseudomonas aeruginosa* Fur. *Future Microbiol* 13, 853-856. doi: 10.2217/fmb-2018-0081.
- Visca, P., Imperi, F., and Lamont, I.L. (2007). Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* 15(1), 22-30. doi: 10.1016/j.tim.2006.11.004.
- Visca, P., Leoni, L., Wilson, M.J., and Lamont, I.L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* 45(5), 1177-1190. doi: 10.1046/j.1365-2958.2002.03088.x.
- Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., and Iglewski, B.H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 185(7), 2080-2095. doi: 10.1128/jb.185.7.2080-2095.2003.
- Wahjudi, M., Papaioannou, E., Hendrawati, O., van Assen, A.H.G., van Merkerk, R., Cool, R.H., et al. (2011). PA0305 of *Pseudomonas aeruginosa* is a quorum quenching acylhomoserine lactone acylase belonging to the Ntn hydrolase superfamily. *Microbiology (Reading)* 157(Pt 7), 2042-2055. doi: 10.1099/mic.0.043935-0.
- War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal Behav* 7(10), 1306-1320. doi: 10.4161/psb.21663.

- Weiland-Brauer, N., Kisch, M.J., Pinnow, N., Liese, A., and Schmitz, R.A. (2016). Highly Effective Inhibition of Biofilm Formation by the First Metagenome-Derived AI-2 Quenching Enzyme. *Front Microbiol* 7, 1098. doi: 10.3389/fmicb.2016.01098.
- West, S.E., Sample, A.K., and Runyen-Janecky, L.J. (1994). The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol* 176(24), 7532-7542. doi: 10.1128/jb.176.24.7532-7542.1994.
- Whitchurch, C.B., Hobbs, M., Livingston, S.P., Krishnapillai, V., and Mattick, J.S. (1991). Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* 101(1), 33-44. doi: 10.1016/0378-1119(91)90221-v.
- Wick, M.J., Frank, D.W., Storey, D.G., and Iglewski, B.H. (1990). Structure, function, and regulation of *Pseudomonas aeruginosa* exotoxin A. *Annu Rev Microbiol* 44, 335-363. doi: 10.1146/annurev.mi.44.100190.002003.
- Wilhelm, S., Gdynia, A., Tielen, P., Rosenau, F., and Jaeger, K.E. (2007). The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. *J Bacteriol* 189(18), 6695-6703. doi: 10.1128/JB.00023-07.
- Williams, P., and Camara, M. (2009). Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 12(2), 182-191. doi: 10.1016/j.mib.2009.01.005.
- Williams, P., Winzer, K., Chan, W.C., and Camara, M. (2007). Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* 362(1483), 1119-1134. doi: 10.1098/rstb.2007.2039.
- Winsor, G.L., Griffiths, E.J., Lo, R., Dhillon, B.K., Shay, J.A., and Brinkman, F.S. (2016). Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 44(D1), D646-653. doi: 10.1093/nar/gkv1227.
- Wolfender, J.L., Nuzillard, J.M., van der Hoof, J.J.J., Renault, J.H., and Bertrand, S. (2019). Accelerating Metabolite Identification in Natural Product Research: Toward an Ideal Combination of Liquid Chromatography-High-Resolution Tandem Mass Spectrometry and NMR Profiling, in Silico Databases, and Chemometrics. *Anal Chem* 91(1), 704-742. doi: 10.1021/acs.analchem.8b05112.
- Xiao, G., Deziel, E., He, J., Lepine, F., Lesic, B., Castonguay, M.H., et al. (2006). MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands. *Mol Microbiol* 62(6), 1689-1699. doi: 10.1111/j.1365-2958.2006.05462.x.
- Zambelloni, R., Marquez, R., and Roe, A.J. (2015). Development of antivirulence compounds: a biochemical review. *Chem Biol Drug Des* 85(1), 43-55. doi: 10.1111/cbdd.12430.
- Zaynab, M., Fatima, M., Abbas, S., Sharif, Y., Umair, M., Zafar, M.H., et al. (2018). Role of secondary metabolites in plant defense against pathogens. *Microb Pathog* 124, 198-202. doi: 10.1016/j.micpath.2018.08.034.
- Zhu, K., and Rock, C.O. (2008). RhlA converts beta-hydroxyacyl-acyl carrier protein intermediates in fatty acid synthesis to the beta-hydroxydecanoyl-beta-hydroxydecanoate component of rhamnolipids in *Pseudomonas aeruginosa*. *J Bacteriol* 190(9), 3147-3154. doi: 10.1128/JB.00080-08.
- Zimmermann, S., Wagner, C., Muller, W., Brenner-Weiss, G., Hug, F., Prior, B., et al. (2006). Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* 74(10), 5687-5692. doi: 10.1128/IAI.01940-05.

RÉSUMÉ ÉTENDU DE LA THÈSE

SYNTHÈSE DE LA THÈSE (Français)

La résistance bactérienne aux antibiotiques est un des plus grands problèmes auquel doit faire face la médecine du 21^{ème} siècle. A l'heure actuelle, l'expansion préoccupante de ce phénomène entraîne plus de 700 000 décès par an, chiffre qui pourrait atteindre 10 millions en 2050 si rien n'est entrepris. Au-delà de ce constat tragique, le coût économique de cette menace est réel et a été estimé à plus 100 000 Milliards de dollars pour les 30 ans à venir (O'Neill, 2016). L'Organisation Mondiale de la Santé s'est saisie de ce problème et a adopté en 2015 un plan d'action mondial pour combattre la résistance. Elle a aussi pré-alerte les pouvoirs publics en 2017 en publiant une liste d'agents pathogènes prioritaires les plus menaçants pour la santé humaine, afin d'essayer de promouvoir la recherche-développement de nouveaux antibactériens pour les combattre (<https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>). En effet, la France demeure un pays sur-consommateur d'antibiotiques et apparaît comme l'un des pays européens les plus touchés par la résistance aux antibiotiques. La multi-résistance aux antibiotiques est donc un problème majeur de santé publique dans notre pays (ANR, AAP PIA « Antibiorésistance 2020 »). De plus, si les antibiotiques existants perdent leur effet, les faibles investissements du secteur pharmaceutique dans ce domaine font qu'une seule famille d'antibactériens a été découverte ces trente dernières années, efficace uniquement contre les bactéries à Gram positif. Ainsi, la communauté scientifique se trouve dans l'urgence et la nécessité d'identifier de nouveaux agents antibactériens capables de combattre les pathogènes résistants et/ou de développer de nouvelles stratégies de lutte contre ces germes.

INTRODUCTION

I. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa est une γ -protéobactérie, aérobie stricte, mobile par un flagelle polaire et dotée d'un génome de grande taille (6,3 Mpb). Ce bacille à Gram négatif, mésophile est capable de s'adapter à de nombreux environnements tels que l'eau, le sol et l'air, grâce à un métabolisme très versatile et une régulation sophistiquée de l'expression de ses gènes (Pirnay *et al.*, 2009 ; Selezska *et al.*, 2012). Longtemps considéré comme ubiquitaire, ce germe est cependant rarement isolé dans des environnements vierges, suggérant que cette bactérie est en fait retrouvée dans des environnements associés à l'activité humaine (Crone *et al.*, 2020). Ses capacités adaptatives lui permettent de répondre rapidement à des modifications des conditions de son environnement, à l'origine en particulier, de son développement en mode de vie planctonique ou communautaire, associé à des infections de type aiguë ou chronique, respectivement. Cette bactérie peut en effet provoquer un large éventail d'infections graves des voies respiratoires, urinaires, cutanées, auriculaires, oculaires, neuro-méningées (Oliveira *et al.*, 2020). Elle est également retrouvée dans l'arbre trachéo-bronchique des patients atteints de mucoviscidose, provoquant l'aggravation des symptômes liés à cette maladie lorsqu'elle est durablement installée (Lyczak *et al.*, 2000 ; 2002, Haussler, 2004, Starkey *et al.*, 2009).

En France et dans le monde, cette bactérie est donc un enjeu majeur de santé publique : elle représente la 2^{ème} cause de pneumopathies nosocomiales et est la 3^{ème} espèce de bacille à Gram négatif la plus fréquemment identifiée au cours de bactériémie (Pachori *et al.*, 2019). Elle est responsable d'infections chez 60 à 80% de patients atteints de mucoviscidose (Acosta *et al.*, 2020). La prévalence des infections à *P. aeruginosa* en service de réanimation et soins intensifs est de 15,3% (Hoang *et al.*,

2018). Ce pathogène opportuniste peut en effet se révéler redoutable et difficile à maîtriser pour des personnes fragilisées ou immunodéprimées. Il est résistant de manière intrinsèque à de nombreux antibiotiques et antiseptiques, grâce notamment à un arsenal d'enzymes de dégradation et de pompes d'efflux ainsi qu'à une capacité à former un biofilm résistant. Ce germe est également capable d'acquérir rapidement de nouvelles résistances sous pression de sélection (Breidenstein *et al.*, 2011; Poole, 2011). L'ensemble de ces éléments a conduit l'Organisation Mondiale de la Santé (OMS) à classer *P. aeruginosa* comme priorité critique dans sa liste de pathogènes contre lesquels la recherche et le développement de nouvelles thérapies doivent être accélérés (OMS, 2017). Près de 14% des isolats de *P. aeruginosa* étaient classés MDR (multidrug-resistant) en 2015 (Antimicrobial resistance surveillance in Europe, European Centre for Disease Prevention and Control, 2015).

1. Infections aiguës et infections chroniques

Les infections dues à *P. aeruginosa* sont divisées en deux groupes, les infections aiguës et les infections chroniques, et dépendent de la production de différents facteurs de virulence dont l'expression est liée à des systèmes de perception et de communication complexes (Gooderham et Hancock, 2009).

*Les infections aiguës à *P. aeruginosa* sont des infections invasives et cytotoxiques, liées à la production de nombreux facteurs de virulence. Pour l'essentiel, ils sont sous la dépendance du système de communication bactérienne appelé Quorum Sensing (QS) considéré comme l'un des principaux mécanismes de régulation de l'expression des gènes de virulence (Ben Haj Khalifa *et al.*, 2011). Ce type d'infection repose sur la production de :

- facteurs associés aux cellules tels que les lectines qui inhibent le battement ciliaire des cellules pulmonaires,

- facteurs sécrétés par des systèmes de sécrétion tels que des toxines et des enzymes hydrolytiques entraînant une réponse inflammatoire et une nécrose des tissus infectés pouvant aller jusqu'à une septicémie,

- facteurs exocellulaires qui restent associés aux cellules tout en étant extracellulaires, tels que la pyocyanine, une phénazine tricyclique de couleur bleu-vert qui possède des effets pro-inflammatoires et cytotoxiques en tant qu'agent oxydant ; la pyoverdine, un sidérophore majeur présentant une très forte affinité pour le fer ferrique ; ou encore les rhamnolipides, biosurfactants capables de s'insérer dans les membranes des cellules épithéliales.

*Lors d'infections chroniques, les bactéries sont organisées en une communauté bactérienne complexe, le biofilm. Dans cette structure, les cellules sont incluses dans une matrice extracellulaire comprenant des polysaccharides, rhamnolipides, protéines, acides nucléiques et vésicules, leur conférant en particulier une protection contre le système immunitaire de l'hôte, les agents antimicrobiens et les composés thérapeutiques (Ciofu *et al.*, 2012 ; Hancock, 1998). Outre la production des éléments structuraux de la matrice, la formation de biofilm est associée à l'expression de facteurs d'adhésion et de mobilité, tel que les lectines et les pili de type IV (Davey *et al.*, 2003), dont certains sont également sous le contrôle du QS. Au niveau moléculaire, ce mode de développement est souvent corrélé avec un taux intracellulaire élevé de c-di-GMP et faible d'AMPc (Valentini *et al.*, 2018).

Cette capacité remarquable de *P. aeruginosa* à adapter son comportement en fonction de son microenvironnement est donc un facteur déterminant de sa pathogénicité qui est hautement et finement contrôlé par des systèmes de régulation interconnectés. La pathogénèse de cet organisme ne dépend pas que d'un seul facteur de virulence, mais d'une interaction précise et délicate entre divers facteurs de virulence.

2. Facteurs de virulence

Les facteurs de virulence sont des composants associés aux cellules, exocellulaires ou sécrétés par divers systèmes de sécrétion (Type I, Type II, Type III, Type V et Type VI), qui sont essentiels pour la survie, la colonisation et l'invasion des tissus de l'hôte par *P. aeruginosa*.

La première étape du processus infectieux est la colonisation de la surface épithéliale des cellules hôtes. Cette étape d'adhésion est réalisée grâce à des adhésines telles que des lectines, des pili de type IV ou *fimbriae*, du LPS ou encore des flagelles. Les lectines PA-IL et PA-IIL sont des adhésines (Chemani *et al.*, 2009) montrant de fortes affinités pour le galactose et le fucose, respectivement, sucres que l'on peut retrouver à la surface des cellules procaryotes et eucaryotes (Vallet *et al.*, 2001). Les flagelles et les pili de type IV participent aux mobilités chez *P. aeruginosa*. La mobilité de type swimming correspond à une nage en milieu liquide et est médiée par les flagelles. Le swarming est une mobilité par essaimage qui se produit en milieu semi-solide et fait intervenir 3 composés majeurs, à savoir les flagelles, les pili de type 4 et les rhamnolipides. Enfin, la mobilité de type twitching correspond à un déplacement sur une surface solide et est liée aux pili de type IV. Les flagelles sont impliqués dans la pathogénie des bactéries puisqu'ils contribuent non seulement à la dissémination de la bactérie, mais favorisent également l'invasion et la colonisation de l'hôte. Le pilus de type IV est la principale adhésine de *P. aeruginosa* (Déziel *et al.*, 2001). Ancré dans la membrane interne et émergeant de la membrane externe, il est constitué de monomères de pilines. La capacité de rétractation du pilus confère à la bactérie une mobilité de type « twitching » (Whitchurch *et al.*, 1991) et de type « swarming » (Kohler *et al.*, 2000). Plusieurs études réalisées *in vitro* et *in vivo* sur des modèles animaux, ont révélé que les pili sont des structures indispensables

à l'adhésion et à l'invasion de *P. aeruginosa* confirmant leur caractère pro-virulent vis-à-vis de l'hôte (Comolli *et al.*, 1999). Le lipopolysaccharide (LPS) est un lipoglycane ancré dans le feuillet externe de la membrane externe des bacilles à Gram négatif. A côté de son action stabilisatrice de la membrane, le LPS joue un rôle dans le pouvoir pathogène des *Pseudomonas* et possède des propriétés antigéniques (Oliveira & Reygaert 2020).

Après cette étape de colonisation, le processus infectieux évolue, soit vers une phase d'infection chronique caractérisée par la formation de biofilms, soit vers une phase d'infection aiguë, caractérisée par une production majeure de facteurs de virulence extracellulaires dépendant de la signalisation cellulaire par le QS, qui endommagent sévèrement les tissus infectés et facilitent l'invasion du système sanguin et la dissémination bactérienne (Van Delden *et al.*, 1998).

Les phénazines sont des molécules hétérocycliques azotées qui sont toxiques pour les cellules eucaryotes et procaryotes. Bien que la structure centrale de la phénazine soit similaire dans différentes phénazines, la nature et la position des substituants sur le cycle hétérocyclique déterminent principalement leur couleur et leurs propriétés biologiques (Price-Whelan *et al.*, 2006). Deux groupes de gènes biosynthétiques de la phénazine peuvent être trouvés dans le génome de *P. aeruginosa*, *phz1* et *phz2* (Mavrodi *et al.*, 2001). Ceux-ci semblent redondants. Cependant, ils diffèrent par le fait que le cluster *phz1*, mais pas *phz2*, contient les gènes *phzM* et *phzS*. Un autre gène impliqué dans la biosynthèse de la phénazine (*phzH*) est situé à une position éloignée (par rapport aux deux clusters *phz*) dans le génome. Les produits des gènes *phzA-G* sont impliqués dans la biosynthèse de l'acide phénazine-1-carboxylique (PCA) à partir de l'acide chorismique. Le PCA peut ensuite être converti en phénazine-1-carboxamide (PCN), 1-hydroxyphénazine (1-HP) et acide 5-méthylphénazine-1-carboxylique bêtaïne (MPCAB) par l'action des

enzymes PhzH, PhzS et PhzM, respectivement. De plus, le MPCAB peut être converti en pyocyanine via PhzS. Bien que l'on pense que les phénazines peuvent contribuer à la virulence de *P. aeruginosa* dans les infections aiguës et chroniques, les groupes de gènes *phz1* et *phz2* ont été trouvés pour contribuer différemment à la production de phénazine au cours de différents modes de croissance de *P. aeruginosa* (Recinos *et al.*, 2012). Plus spécifiquement, les gènes *phz1* sont davantage exprimés au cours de la croissance planctonique de *P. aeruginosa*, tandis que les gènes du groupe *phz2* contribuent presque exclusivement à la biosynthèse de phénazines dans les biofilms. Les phénazines médient leur activité toxique sur les eucaryotes et procaryotes principalement en provoquant un stress oxydatif. Les phénazines réduites sont capables de réagir avec l'oxygène, pour produire des espèces réactives de l'oxygène (superoxyde et peroxyde d'hydrogène), causant ainsi des dommages oxydatifs aux cellules hôtes (Muller, 2002). Enfin, la pyocyanine est un pigment bleu, conférant son nom de « bacille pyocyanique » à *P. aeruginosa* due à la couleur typique et caractéristique des cultures et infections liées à *P. aeruginosa* (Fothergill *et al.*, 2007). La pyocyanine est le métabolite de type phénazine le plus étudié et est couramment considéré comme un marqueur d'activité du quorum sensing (QS) (Tomas *et al.*, 2018 ; Vilaplana & Marco, 2020). La pyocyanine inactive la catalase des cellules eucaryotes, augmentant le taux de radicaux libres et le stress oxydatif, ce qui conduit à la nécrose tissulaire (O'Malley *et al.*, 2003). Elle joue également un rôle dans l'acquisition du fer (Cornelis et Dingemans, 2013) et est également impliqué dans la suppression de la réponse immunitaire et l'apoptose des neutrophiles dans la cellule hôte (Allen *et al.*, 2005; Kipnis *et al.*, 2006). Elle peut également inhiber la respiration cellulaire ou la fonction ciliaire des cellules pulmonaires (Sorensen et Joseph, 1993). La pyocyanine peut également fonctionner comme une molécule signal puisqu'elle contrôle l'expression de plusieurs gènes impliqués dans l'efflux de drogues, les processus redox ainsi que l'acquisition du fer (Dietrich *et al.*, 2006).

Les rhamnolipides sont des biosurfactants de nature glycolipidique qui consistent en un (mono-rhamnolipide) ou deux groupements rhamnose (di-rhamnolipide) liés entre eux par une liaison α -1,2-glycosidique, et une à trois chaînes d'acides gras d'une longueur comprise entre 8 et 16 atomes de carbone liées par une liaison ester. Les groupements rhamnose sont liés aux acides gras par liaison O-glycosidique (Abdel-Mawgoud *et al.*, 2010). De nombreuses variations existent dans la structure des rhamnolipides, en fonction du nombre de groupements rhamnose, du nombre et de la longueur de chaîne d'acide gras, ou encore du nombre de liaisons insaturées dans la chaîne d'acide gras. Ces différentes combinaisons conduisent à l'identification de plus de 60 structures de rhamnolipides différentes parmi lesquels les di-rhamnolipides semblent être les plus abondamment produits (Abdel-Mawgoud *et al.*, 2010). Ils sont synthétisés par les enzymes RhlA, RhlB (mono-rhamnolipide), et RhlC (di-rhamnolipide) (Zhu et Rock, 2008). Un opéron de deux gènes *rhlAB* codant pour les protéines responsables de la synthèse des rhamnolipides est suivi des deux gènes régulateurs *rhlR* et *rhlI*, dont l'expression est positivement régulée par le système Rhl du QS. Les rhamnolipides sont impliqués dans la motilité de type swarming de *P. aeruginosa* qui est également dépendante du QS (Köhler *et al.*, 2000). Les rhamnolipides fonctionnent comme des agents mouillants, réduisant la tension superficielle entourant la colonie bactérienne, facilitant ainsi la migration des cellules de *P. aeruginosa* au travers d'une surface semi-solide (Caiazza *et al.*, 2005). De plus, les rhamnolipides sont impliqués dans la dispersion des cellules des biofilms de *P. aeruginosa* (Boles *et al.*, 2005) et dans le maintien des canaux en eau libre dans les biofilms matures (Davey *et al.*, 2003). Enfin, il a été constaté que les rhamnolipides sont capables de lyser les neutrophiles polynucléaires et d'entourer les biofilms de *P. aeruginosa* pour les protéger contre l'approche de ces leucocytes (Alhede *et al.*, 2009).

Comme tous les organismes, les bactéries ont besoin de fer, qui agit comme cofacteur de leurs enzymes redox-dépendantes. Dans la plupart des environnements, le taux de fer soluble est trop faible pour être acquis par diffusion passive par la cellule (Griffiths, 1987). Les bactéries ont alors élaboré différentes stratégies pour faire face à cette carence, parmi lesquelles la sécrétion de molécules chélatant le fer, nommée sidérophores (Guerinot, 1994). Ces sidérophores présentent une forte affinité pour le fer ferrique, et les complexes ferrisidérophores ainsi formés seront ensuite captés par des récepteurs de membrane externe, qui vont permettre leur passage dans le périplasme via un transport actif TonB-dépendant, avant d'être transloqués dans le cytoplasme bactérien, puis réduits en fer ferreux (Schalk *et al.*, 2002; Cornelis 2010; Cornelis *et al.*, 2009). La pyoverdine, pigment de couleur verte, est le premier sidérophore identifié chez *P. aeruginosa* et chélate le fer ferrique Fe³⁺ à une stœchiométrie de 1:1. La pyochéline quant à elle chélate le fer à une stœchiométrie de 2:1 et à une affinité plus faible que la pyoverdine car elle peut se complexer avec une variété d'autres métaux, comme l'aluminium, le cuivre ou encore le nickel (Cornelis, 2008). Ces sidérophores sont notamment régulés par de nombreux facteurs sigma à fonction extracytoplasmique (Facteurs σ ECF) (Chevalier *et al.*, 2018), les petits ARNs PrrF1 et PrrF2 et le répresseur majeur Fur. Ce répresseur, en absence de fer, ne se fixe pas sur ses séquences régulatrices, et permet la transcription et la production de nombreux systèmes d'acquisition du fer, et donc de sidérophores, dont certains sont des facteurs de virulence chez *P. aeruginosa*, comme la pyoverdine et la pyochéline (Visca *et al.*, 2002 ; Visca et Imperi, 2018). L'importance de ces sidérophores dans la toxicité bactérienne a largement été démontrée, les mutants déficients en sidérophores étant invariablement moins virulents (Ratledge & Dover, 2000). La pyoverdine présente une activité cytotoxique envers les leucocytes humains (Becerra *et al.*, 2001). Outre son rôle de chélateur de fer, la pyoverdine contrôle sa synthèse en plus de celle de facteurs de virulence sécrétés tels que l'endoprotéase et l'exotoxine A (Gaines *et al.*, 2007).

P. aeruginosa possède une grande variété de toxines et d'enzymes sécrétées, dont la sécrétion est réalisée par des systèmes de sécrétion de Type (SST) 1, 2, 3, 5 et 6 (Bleves *et al.*, 2010).

- Le SST1 est un ABC-transporteur qui est connu pour exporter au moins 3 substrats parmi lesquels la protéine de fonction inconnue AprX, l'hémophore HasA qui permet de piéger le fer contenu dans l'hème, et la métalloprotéase alcaline AprA, qui présente une activité maximale à pH alcalin et qui est capable de dégrader différents intervenants du système immunitaire tels que des composés du complément, les interférons- γ , le TNF- α ou encore certaines interleukines (Duong *et al.*, 2001).

- Le SST2 sécrète la majorité des enzymes hydrolytiques et des toxines, tels que l'exotoxine A, les élastases LasA et LasB ou encore la protéase PrpL (Filloux *et al.*, 2011). L'exotoxine A est une toxine qui cible la synthèse de protéines entraînant la mort de la cellule cible par nécrose (Wick *et al.*, 1990). Sa synthèse est régulée par le QS et par le facteur sigma à fonction extracytoplasmique PvdS (Visca *et al.*, 2007 ; Chevalier *et al.*, 2019). Les élastases LasA (également appelée staphylolysine) et LasB, sont toutes deux capables de dégrader l'élastine, avec un effet synergique. LasB est également capable de dégrader le collagène ou encore de cliver les immunoglobulines de type A et G (Bainbridge & Fick, 1989; Gellatly & Hancock, 2013; Heck *et al.*, 1990; Mariencheck *et al.*, 2003; Thibodeau et Butterworth, 2013). Ces enzymes permettent également les jonctions serrées favorisant ainsi l'invasion de l'épithélium pulmonaire eucaryotes (Nomura *et al.*, 2014). Ces 2 protéines sont sous le contrôle du QS (Popat *et al.*, 2008). La protéase IV (ou Piv ou PrpL) est une endoprotéase impliquée dans la dégradation du fibrinogène, de l'immunoglobuline G, de la plasmine, du plasminogène et du complément (Malloy *et al.*, 2005; Matsumoto, 2004). PrpL est également impliquée dans le clivage de la lactoferrine et

la transferrine, permettant d'en libérer le fer. Sa biosynthèse est régulée par PvdS et le QS (Chevalier *et al.*, 2019).

- Le SST3 ou injectisome est un système qui délivre des toxines (exoenzymes S, T, U, Y) directement dans le cytoplasme d'une cellule eucaryote après contact avec la bactérie. Les exoenzymes ExoS et ExoT possèdent chacune une activité inhibant les GTPases de la cellule cible, ainsi qu'une activité ADP-ribosylation. L'exoenzyme ExoY est une adénylate cyclase permettant l'élévation du taux d'AMPc dans la cellule cible et l'exoenzyme ExoU est une phospholipase. L'ensemble de ces toxines conduit à la réorganisation du cytosquelette et à la nécrose de la cellule cible (Barbieri et Sun, 2004 ; Hauser 2009).

- Le SST5 est unique dans le sens où il utilise la machinerie Sec pour transporter des substrats dans le périplasme, puis ces substrats ou autotransporteurs sont capables de s'auto-transporter au travers de la membrane externe (Pena *et al.*, 2019). L'auto-transporteur EstA a été montré pour être nécessaire à la production des rhamnolipides (Wilhelm *et al.*, 2007).

- *P. aeruginosa* dispose de 3 SST6 notés H1-SS T6 à H3-SS T6. Ce sont des injectisomes dont le H1-SST6 cible uniquement des cellules procaryotes, alors que les H2- et H3-SST6 ciblent à la fois des cellules procaryotes et eucaryotes en y injectant directement des toxines (Sana *et al.*, 2016). Ces systèmes sont impliqués dans la colonisation d'une niche écologique et dans la pathogénie de la bactérie dans le cas des H2- et H3-SST6.

3. Le Quorum sensing (QS)

P. aeruginosa est capable de contrôler la production de facteurs de virulence grâce à un système de communication intercellulaire bactérien, le quorum sensing (QS). Le QS est un mécanisme de communication de cellule à cellule qui permet aux bactéries de coordonner l'expression génique de manière dépendante de la densité

cellulaire. Ces systèmes permettent ainsi la synchronisation du métabolisme et de la physiologie des cellules en réponse à un environnement donné. La communication entre bactéries est assurée par de petites molécules nommées autoinducteurs, que les bactéries produisent et sécrètent de façon continue en très faible quantité. Lorsque la densité cellulaire, et donc la concentration de ces autoinducteurs atteint un seuil critique (le quorum), une cascade de régulation se déclenche, permettant in fine la régulation d'un grand nombre de gènes de façon coordonnée, ainsi qu'une très forte augmentation de la production d'autoinducteurs. La plupart de ces systèmes s'auto-induisent une fois le quorum atteint par une boucle de rétroaction positive (LaSarre et Federle, 2013).

Chez *P. aeruginosa*, trois systèmes de QS (Las, Rhl et PQS) ont été décrits et contrôlent l'expression des gènes de virulence de manière hiérarchique (Moradali *et al.*, 2017 ; Turkina and Vikström, 2019). Les systèmes Las et Rhl reposent sur la production de N-acyl-homosérine lactones (AHL) comme molécules signal, tandis que le système PQS répond aux 2-alkyl-4-quinolones (HAQ) (Schuster et Greenberg, 2006; Williams Et Camara, 2009). A forte densité cellulaire, les concentrations extracellulaires en AHL augmentent et un seuil critique intracellulaire est atteint permettant aux AHL de se fixer sur leur régulateur de type LuxR respectif. Le complexe néoformé interagit avec des séquences d'ADN cibles et augmente ou bloque la transcription de certains gènes, régulant ainsi l'expression des gènes dépendants du QS. La concentration en AHL est maximale entre les phases de croissance exponentielle et stationnaire, contrairement au système PQS qui est actif en phase stationnaire, quand la concentration en PQS libérée est maximale (McKnight *et al.*, 2000). Il semble cependant que le QS ne soit pas uniquement dépendant de la densité de population bactérienne, mais qu'il serait également induit par des facteurs environnementaux (van Delden *et al.*, 2001; Williams & Camara, 2009).

Les AHL sont constituées d'acides gras dont la taille est comprise entre 4 à 18 atomes de carbone, liés par une liaison peptidique à une homosérine lactone. La partie acide gras peut être modifiée via un substituant 3-oxo ou 3-hydroxy (Hirakawa et Tomita, 2013). Les AHL sont synthétisées par des enzymes de la famille LuxI, tout en étant détectées par des régulateurs transcriptionnels de la famille LuxR (Fuqua *et al.*, 1996). Le système Las produit et répond à la 3-oxo-C₁₂-HSL (N-(3-oxo-dodécanoyl)-L-homosérine lactone), tandis que le système Rhl produit et est activé par la C₄-HSL (N-butyryl-L-homosérine lactone). Lorsqu'une population de *P. aeruginosa* atteint une certaine densité à laquelle la concentration seuil d'un certain AHL est atteinte, l'AHL formera un complexe avec un régulateur LuxR, activant ainsi la transcription d'un certain nombre de gènes de virulence, ainsi que la sienne et celle du gène *luxI* correspondant (Pesci *et al.*, 1997; Schuster and Greenberg, 2006; Turkina and Vikström, 2019). Le complexe LasR/3-oxo-C₁₂-HSL exerce un rétrocontrôle positif sur *lasI* (Seed *et al.*, 1995), suggérant que lorsque la concentration seuil de 3-oxo-C₁₂-HSL est atteinte, elle ne cesse d'augmenter jusqu'à l'intervention d'un autre facteur. *P. aeruginosa* est en effet capable de dégrader les 3-oxo-C₁₂-HSL pendant la phase stationnaire de croissance, par l'intervention d'une HSL acylase et d'une HSL lactonase (Huang *et al.*, 2003). Le transfert des 3-oxo-C₁₂-HSL à travers les membranes vers le milieu extérieur pourrait faire intervenir la pompe à efflux MexAB/OprM (Evans *et al.*, 1998; Pearson *et al.*, 1999), bien que l'implication de cette pompe dans l'efflux de ces autoinducteurs est controversée (Alcalde-Rico *et al.*, 2020). Des études de radio-marquage ont révélé que les C₄-HSL diffusaient librement à travers la membrane, contrairement aux 3-oxo-C₁₂-HSL (Pearson *et al.*, 1999).

Le troisième système de communication est basé sur l'utilisation d'une quinolone comme molécule de communication, la 2-heptyl-3-hydroxy-4-quinolone, aussi nommée Pseudomonas Quinolone Signal (PQS). Le régulateur transcriptionnel

PqsR (MvfR) contrôle l'expression des opérons *pqsABCDE* et *phnAB* codant pour les enzymes impliquées dans la biosynthèse des molécules de signalisation qui appartiennent à la famille des 4-hydroxy-2-alkylquinolines (HAQ). L'opéron *phnAB* code les sous-unités α et β de l'antranilate synthase PhnAB, responsable en partie de la biosynthèse de l'acide anthranilique à partir de l'acide chorismique (Gallagher *et al.*, 2002). La voie des kynurénines permet également la production de l'acide anthranilique (Farrow *et al.*, 2007). L'opéron *pqsABCDE* code les enzymes PqsA, PqsB, PqsC, PqsD et PqsE qui sont nécessaires à la biosynthèse des HAQ, à exception de PqsE. PqsA, PqsB, PqsC, PqsD catalysent la conversion de l'antranilate en 2-heptyl-4-quinolone (HHQ). HHQ est ensuite hydroxylé par PqsH, permettant de produire PQS. Cette molécule se fixe sur PqsR (ou MvfR) pour activer la transcription des gènes sous sa dépendance (Lee et Zhang, 2014). PqsE semble être impliquée dans la régulation de l'expression de nombreux facteurs de virulence contrôlés par le régulon de PqsR, comme la pyocyanine, les rhamnolipides, lectines, et les élastases (Gallagher *et al.*, 2002; Diggle *et al.*, 2003 ; Rampioni *et al.*, 2016; Schütz et Empting, 2018). HHQ joue également un rôle de signal (Kim *et al.*, 2010). Ce système est le plus souvent présenté comme un modulateur des deux autres systèmes de QS, car, en plus de s'autoréguler et de réguler l'opéron *pqsABCDE*, PqsR est aussi impliqué dans la régulation de *lasR* et *rhlR* (Lee et Zhang, 2014; Maura *et al.*, 2016). PQS et PqsR régulent jusqu'à 140 gènes, dont la majorité sont co-régulés par Rhl (Déziel *et al.*, 2005).

Plus de cinquante molécules appartenant à la famille des HAQ ont été identifiés chez *P. aeruginosa* (Lépine *et al.*, 2004). Les HAQ les plus abondantes produites par *P. aeruginosa* sont celles qui portent une chaîne aliphatique à sept carbones, soient le HHQ (4-hydroxy-2-heptylquinoline), le PQS (3,4-dihydroxy-2-heptylquinoline, ou Pseudomonas Quinolone Signal) et le HQNO (4-hydroxy-2-heptylquinoline N-oxyde). Néanmoins, seulement le HHQ et le PQS sont connus comme auto-

inducteurs et donc considérés comme des molécules signal parmi l'ensemble des quinolones produites par *P. aeruginosa* (Diggle *et al.*, 2001). PQS est une molécule relativement hydrophobe, très peu soluble dans les solvants aqueux (Calfee *et al.*, 2005) et possédant une haute affinité pour les membranes riches en lipides (Lepine *et al.*, 2003). Ceci suggère que *P. aeruginosa* a développé un moyen pour solubiliser cette molécule. Les rhamnolipides contribuent à la solubilisation de PQS et augmentent l'assimilation de PQS dans les cellules (Calfee *et al.*, 2005). Mais PQS est majoritairement retrouvé dans les vésicules membranaires qui peuvent contenir jusqu'à 86% de la production totale de PQS par *P. aeruginosa* (Mashburn & Whiteley, 2005). Il a également été rapporté que PQS forme des complexes avec le fer (III), ce qui indique qu'en plus de sa fonction de molécule signal, il joue un rôle de chélateur du fer pour accélérer la délivrance de fer associée aux sidérophores (Diggle *et al.*, 2007; Lin *et al.*, 2018; Maura *et al.*, 2016). Le PQS intervient également dans la biogenèse des vésicules (Cooke *et al.*, 2019).

4. Infections de type chronique et biofilms

Lorsqu'elles ne sont pas éradiquées pendant la phase de colonisation, les bactéries peuvent s'adapter à leur environnement et développer un mode de vie sessile et communautaire, le biofilm (Van Delden *et al.*, 1998). Sous cette forme, *P. aeruginosa* adopte un phénotype beaucoup moins agressif en cessant de produire les facteurs associés à sa toxicité aiguë, et se protège en particulier contre la dessiccation, les antimicrobiens et les effecteurs du système immunitaire de l'hôte (Reichardt et Parsek, 2019). Ce biofilm induit une inflammation persistante chez l'hôte et provoque alors généralement une infection de type chronique, qui peut être à l'origine d'une destruction progressive des tissus. Les infections chroniques à *P. aeruginosa* sont fréquentes chez les personnes ayant des problèmes respiratoires, comme les broncho-pneumopathies chroniques obstructives et les bronchectasies,

mais surtout chez les patients atteints de mucoviscidose (Gellatly and Hancock, 2013; Mulcahy *et al.*, 2014). Bien que le biofilm puisse se former sur des tissus natifs, les infections en biofilm sont cependant majoritairement observées sur des dispositifs médicaux invasifs tels que les sondes urinaires, les matériaux ostéo-articulaires ou encore les sondes endotrachéales par exemple (Lebeaux *et al.*, 2014). Les biofilms sont impliqués dans 65% des infections microbiennes et dans plus de 80% des infections chroniques (Jamal *et al.*, 2018).

Un biofilm est une communauté microbienne adhérente sur une surface ou dans laquelle les germes sont adhérents les uns aux autres, englués dans une matrice composée principalement d'exopolysaccharides, d'ADN extracellulaire (eDNA), de protéines et de vésicules de membrane externe (Flemming and Wingender, 2010 ; Mulcahy *et al.*, 2014). La formation de biofilms implique différents stades de formation: (I) l'attachement de la bactérie à la surface de l'hôte ; (II) l'attachement irréversible aux surfaces et le développement de la micro-colonie (III) ; la différenciation précoce du biofilm sous la forme de micro-colonies et le développement de son architecture ; (IV) la maturation du biofilm et (V) la dispersion du biofilm formé (Stoodley *et al.*, 2002). La formation des biofilms est médiée en partie par le QS (Davies, 1998; Diggle *et al.*, 2006) et dépend de facteurs tels que la concentration en fer (Bollinger *et al.*, 2001), et l'état nutritionnel (Sauer *et al.*, 2004).

La formation et le maintien de l'intégrité de l'architecture du biofilm sont assurés par une matrice exocellulaire. Elle limite le taux de pénétration et de diffusion de certains antibiotiques et crée un environnement stratifié dont le cœur présente peu ou pas d'oxygène, une faible croissance bactérienne et une faible activité métabolique (Anwar et Costerton, 1990). Par conséquent, les gradients d'oxygène et de nutriments induisent le développement de sous-populations

bactériennes avec des niveaux variables de sensibilité aux antibiotiques (Jefferson *et al.*, 2005). Elle est composée principalement d'exopolysaccharides (EPS), mais aussi de protéines, de lipides, d'ADN extracellulaire, de rhamnolipides et de vésicules de membrane externe (Mann & Wozniak, 2012). *P. aeruginosa* produit au moins trois EPS (alginate, Pel et Psl) qui sont déterminants pour la stabilité de la structure du biofilm. Pel et Psl servent de structure primaire d'échafaudage pour le développement du biofilm, Pel étant impliqué dans l'initiation des biofilms et Psl, dans la maturation des biofilms (Colvin *et al.*, 2011 ; Ma *et al.*, 2006). *P. aeruginosa* sécrète plutôt Psl pour coloniser les surfaces () et a été démontré surproduire l'alginate pendant la croissance dans certaines conditions physiques telles qu'un faible cisaillement des fluides (Crabbe *et al.*, 2008;) ou à la suite de l'acquisition de mutations au cours de la croissance dans l'environnement pulmonaire de la mucoviscidose Pedersen, 1992). Les alginates peuvent contribuer à la stabilité structurelle et à la protection du biofilm, en réponse à certaines conditions environnementales (stress physique, Crabbe *et al.*, 2008 ; 2010), et chez des patients atteints de mucoviscidose (Feliziani *et al.*, 2010 ; Lyczak *et al.*, 2002). En effet, *P. aeruginosa* peut subir la conversion mucoïde suite à l'apparition de mutations dans le gène *mucA* principalement, conduisant à une hyperactivité du facteur sigma à fonction extracytoplasmique AlgU, qui contrôle en particulier l'expression des alginates (Ramsey and Wozniak, 2005 ; Chevalier *et al.*, 2019). Ces polymères d'acides glucuronique et mannuronique favorisent l'attachement irréversible du pathogène aux cellules épithéliales pulmonaires et inhibent la fonction de clairance (Ramsey and Wozniak, 2005). Les appendices extracellulaires de *P. aeruginosa*, comme les flagelles, les pili de type IV et les fimbriae, sont également considérés comme des composants de la matrice du biofilm jouant un rôle adhésif dans les interactions cellule-surface (Rasamiravaka *et al.*, 2015 ; Ryder *et al.*, 2007 ; Ben Haj Khalifa *et al.*, 2011; Gellatly and Hancock, 2013). En outre, les biosurfactants rhamnolipides sont nécessaires pour le remodelage de la matrice de biofilm (Pamp

et Tolker-Nielsen, 2007) et favorisent les mécanismes de dispersion qui permettent la libération de cellules planctoniques (Boles *et al.*, 2005). La production de la matrice exocellulaire est en partie sous la dépendance du taux intracellulaire de c-di-GMP, qui, en forte concentration favorise la production de la matrice extracellulaire alors qu'un taux faible favorisera la mobilité et par conséquent le mode de vie planctonique (Borlee *et al.*, 2010). Une adhésine régulée par le c-di-GMP, CdrA, structure la matrice en reliant Psl à la membrane bactérienne (Borlee *et al.*, 2010).

5. Régulation de la virulence et transition entre les modes de vie planctonique et sessile

L'un des systèmes à deux composants les plus importants pour réguler le passage des infections aiguës aux infections chroniques est le système GacS / GacA. GacS est un senseur qui va s'autophosphoryler en réponse à un signal qui reste inconnu. Le groupement phosphate est ensuite transmis à GacA, qui va activer la transcription de RsmY et RsmZ, deux ARN non codants (ARNnc). Ces ARNnc vont séquestrer RsmA, un répresseur capable de se lier aux ARN messagers (ARNm), atténuant la répression traductionnelle de nombreux facteurs de virulence associés à une infection chronique, tout en réprimant les facteurs de virulence associés à une infection aiguë (Heurlier *et al.*, 2004 ; Kay *et al.*, 2006). En effet, ce répresseur, sous forme libre, va réguler directement des fonctions impliquées dans la formation de biofilm, comme la synthèse des EPS ou encore le système de sécrétion de type VI. Il est également capable d'activer des fonctions impliquées dans la virulence aiguë comme le système de sécrétion de type III, les systèmes d'acquisition du fer, ou encore les mobilités de type « swarming » via les rhamnolipides et les pili de type IV (Brencic et Lory, 2009; Burrowes *et al.*, 2006). La séquestration ou non de cette protéine permet donc d'orienter le mode de vie de la bactérie vers le biofilm ou la forme « libre » (Brencic et Lory, 2009; Burrowes *et al.*, 2006). Il a été démontré que

plusieurs autres kinases interagissent avec le système GacS / GacA. LadS agit en parallèle de GacS et régule positivement les gènes *pel* impliqués dans la formation du biofilm, tout en régulant négativement les gènes impliqués dans le système de sécrétion de type III (Ventre *et al.*, 2006). Au contraire, RetS active l'expression de facteurs de virulence associés à une infection aiguë (Ventre *et al.*, 2006). RetS est capable de former des hétérodimères avec GacS, empêchant l'autophosphorylation de GacS, interférant avec la phosphorylation de GacA. Il en résulte une répression des facteurs de virulence associés à une infection chronique et une expression accrue des facteurs de virulence associés à une infection aiguë (Jimenez *et al.*, 2012 ; Chambonnier *et al.*, 2016). D'autres facteurs influencent l'activité du système GacS/GacA.

L'AMPC (ou adénosine monophosphate) est un messager secondaire produit par des adénylates cyclases, qui est un médiateur majeur de l'expression des facteurs de virulence dans le cadre d'une infection de type aiguë (mode de vie planctonique). L'AMPC se lie au régulateur global Vfr (virulence factor regulator) induisant la production de facteurs de virulence tels que l'exotoxine A, les toxines du SST3, et l'expression des systèmes de QS Las et Rhl. Le c-di-GMP (di-guanosine monophosphate cyclique) est un second messager intracellulaire impliqué dans la transition du mode de vie planctonique au mode sessile (Moradali *et al.*, 2017 ; Valentini et Filloux, 2016), et est inversement corrélé avec celui de l'AMPC. Un mode de vie biofilm sera caractérisé par un haut taux de c-di-GMP et un bas taux d'AMPC, et inversement pour un mode de vie planctonique. Le c-di-GMP est synthétisé à partir de deux guanosine triphosphates (GTP), par des diguanylate cyclases contenant un domaine GGDEF), et dégradé par des phosphodiesterases (domaine EAL). Certaines enzymes possèdent les deux activités. Chez *P. aeruginosa* PAO1, il existe 17 diguanylate cyclases, 5 phosphodiesterases, et 16 enzymes possédant les deux activités (Kulasekara *et al.*, 2005). Il est intéressant de noter que la plupart de

ces enzymes contiennent des domaines protéiques senseurs variés, ou appartiennent à des systèmes protéiques liant des protéines senseurs et ces enzymes synthétisant ou dégradant le c-di-GMP, suggérant que les conditions environnementales affectent le taux de c-di-GMP au sein de la cellule (Ha et O'Toole, 2015).

II. Composés d'origine végétale ciblant la virulence de *P. aeruginosa*

L'augmentation alarmante de la résistance aux antibiotiques a conduit à l'accomplissement de progrès considérables dans la compréhension des mécanismes moléculaires impliqués dans la virulence bactérienne. Le développement de stratégies dites anti-virulence, est apparu comme une alternative intéressante pour la création de nouveaux agents antimicrobiens, puisqu'au lieu de tuer les agents pathogènes, elle tend à réduire leur virulence (Fleitas Martínez *et al.*, 2019). Cette approche présente plusieurs avantages potentiels, notamment l'élargissement du répertoire de cibles bactériennes, la préservation du microbiote endogène et réduction de la pression sélective, ce qui pourrait limiter l'apparition de résistances (Clatworthy *et al.*, 2007 ; Rasko et Sperandio, 2010 ; Totsika, 2016). C'est une alternative prometteuse à l'antibiothérapie traditionnelle pour le traitement des maladies infectieuses, soit seule, soit en association avec un traitement antibiotique (Cegelski *et al.*, 2008; Mühlen et Dersch, 2015; Paul et Leibovici, 2009). Les principaux systèmes de régulation de la virulence font partie des cibles majeures de cette stratégie.

De nombreux efforts ont été consentis pour développer des agents anti-virulence ciblant des facteurs de virulence ou des mécanismes de régulation de la pathogénie. L'inhibition ou atténuation du QS, connue sous le nom de « quorum quenching » (QQ) a attiré beaucoup d'attention en tant que nouvelle approche pour

réduire la pathogénicité bactérienne. En effet, en régulant l'expression des gènes impliqués dans la persistance et la virulence, le QS se présente comme une cible cruciale pour la thérapie anti-virulence. Le QQ est considéré comme une stratégie intéressante de lutte contre les bactéries pathogènes pour différentes raisons (Williams, 2011). Le QQ permettrait de surmonter les mécanismes conventionnels de résistance aux antibiotiques; un seul inhibiteur pourrait avoir des effets multiples, étant capable d'interférer avec la production d'un certain nombre de facteurs de virulence et avec la formation de biofilm; en principe, les facteurs anti-virulence ne doivent pas exercer de pression évolutive sur la population bactérienne ciblée, évitant l'émergence de souches résistantes; en évitant ou en limitant la formation de biofilm, les inhibiteurs de QS pourraient améliorer l'efficacité des antibiotiques existants; à l'exception des membres appartenant à la famille AI-2, la plupart des auto-inducteurs sont spécifiques. Le spectre étroit d'activité des inhibiteurs devrait éviter la perturbation du microbiote bénéfique.

Il est bien connu que les organismes procaryotes et eucaryotes ont développé des mécanismes moléculaires capables d'exercer une activité QQ. L'étude des systèmes de dégradation du signal mis en œuvre par différentes bactéries, plantes et mammifères peut conduire à la conception de nouvelles approches pour éteindre le QS bactérien (Zhang & Dong 2004). Idéalement, un inhibiteur de QS devrait répondre aux critères suivants: il devrait s'agir d'une petite molécule, hautement spécifique, non toxique pour les cellules eucaryotes et chimiquement stable (Kalia, 2013). La composante principale du QS étant la production et la détection de molécules signal, le QQ peut interférer de différentes manières avec ce système, soit au niveau intracellulaire, soit au niveau extracellulaire. L'utilisation de molécules inhibitrices, nommées inhibiteurs du QS (QSI), mimant les molécules signales, permet ainsi d'intervenir au niveau des cellules bactériennes pour inhiber la production ou la perception des molécules signales (Tang & Zhang 2014). D'autres

stratégies ciblent directement les molécules de communication sécrétées dans l'environnement avant qu'elles n'atteignent leur cible, soit en les séquestrant à l'aide d'anticorps (Park *et al.*, 2007) ou de macromolécules comme les cyclodextrines (Kato *et al.*, 2006), soit en les dégradant avec des enzymes spécifiques (ou QQE pour quorum quenching enzyme) (Fetzner 2015). Le QQ présente l'avantage de ne pas affecter la croissance des bactéries, contrairement aux antibiotiques, ce qui minimise nettement la pression de sélection et devrait limiter l'apparition de résistance (Defoirdt *et al.*, 2010). Le QQ représente donc une approche idéale pour la prévention et le traitement des infections bactériennes et pourrait constituer une alternative élégante aux antibiotiques. Différentes stratégies visent à perturber les différentes étapes du système QS, ciblant la production de signaux, les molécules de signalisation et les récepteurs de signaux.

L'utilisation de plantes à des fins médicinales remonte au début de l'espèce humaine. De même que pour les humains et les animaux dans leur écosystème, les plantes ont été continuellement exposées à des bactéries et ont développé plusieurs mécanismes de protection et d'éradication des infections pathogènes, incluant la synthèse de métabolites secondaires en tant qu'antimicrobiens (Dixon, 2001; Mazid *et al.*, 2011). Bien que les plantes soient dépourvues de cellules de défense et d'un système immunitaire comparable aux animaux supérieurs, elles sont capables de reconnaître les éliciteurs ou signaux pathogènes et de répondre de manière appropriée aux stimuli par renforcement de la paroi cellulaire, synthèse de métabolites secondaires, protéines liées à la pathogenèse et enzymes lytiques (War *et al.*, 2012). Ces métabolites secondaires sont des composés organiques qui peuvent être stockés sous des formes inactives, constitutives (phytoanticipines) ou libérés en réponse à une attaque pathogène également connue sous le nom de phytoalexines (Fürstenberg-Hägg *et al.*, 2013). Les phytoanticipines sont présentes avant une attaque pathogène ou produites juste après l'attaque pathogène à partir de

précurseurs préexistants (VanEtten *et al.*, 1994) tandis que les phytoalexines (telles que les terpénoïdes et les polyphénols) sont synthétisées suite à la reconnaissance des éliciteurs issus de l'exposition à une attaque de pathogènes (Hemaiswarya *et al.*, 2008).

Pour compléter cette introduction, nous avons rédigé une revue de la littérature visant à répertorier tous les composés d'origine végétale ayant une activité contre la virulence de *P. aeruginosa*. Cette revue, pour laquelle je serai 1^{er} auteur, est en préparation.

OBJECTIFS DE LA THÈSE

Dans un contexte mondial d'augmentation de la résistance aux antimicrobiens, de nouvelles thérapies alternatives aux antibiotiques sont nécessaires pour lutter contre les infections multi-résistantes. Comme les animaux et les humains, les plantes sont naturellement exposées aux infections bactériennes et peuvent répondre aux stimuli bactériens en produisant des composés phytochimiques ou des métabolites secondaires (Adiloğlu *et al.*, 2016; Zaynab *et al.*, 2018) à des fins défensives, qui pourraient posséder des applications prometteuses pour lutter non seulement contre les phytopathogènes mais aussi contre les pathogènes humaines. Les stratégies alternatives qui se développent dorénavant consistent à interférer avec les éléments qui sont impliqués dans la persistance ou la toxicité de *P. aeruginosa* et ciblent principalement les facteurs de virulence de la bactérie ou les régulateurs qui contrôlent leur biosynthèse (Smith *et al.*, 2017). Contrairement aux antibiotiques actuels, ces molécules ne ciblent pas directement la viabilité bactérienne et le risque de pression de sélection entraînant l'acquisition de résistance devrait donc être fortement amoindri.

Cette thèse s'inscrit dans la thématique principale du Laboratoire de Microbiologie Signaux et Microenvironnement (LMSM EA 4312, Université de Rouen Normandie), à savoir l'étude du Rôle de la communication et des facteurs environnementaux ou eucaryotes dans l'adaptation et la virulence bactériennes ». Elle s'inscrit dans deux champs thématiques majeurs, à savoir « Systèmes senseurs et transducteurs » animé par le Pr S. CHEVALIER et « Communication dans le microbiote humain », animé par le Pr O. LESOUHAITIER. Cette thèse a contribué au développement d'un nouvel axe de recherche au sein du LMSM qui vise à développer de nouvelles stratégies de lutte contre le pathogène opportuniste de l'Homme *P. aeruginosa* à partir d'actifs d'origine végétale (S. CHEVALIER) ou

animale (O. LESOUHAITIER). Ces travaux ont contribué à établir de nouvelles collaborations entre le Pr S. CHEVALIER (LMSM, Université de Rouen Normandie), les Dr Raphaël GROUGNET, Sabrina BOUTEFNOUCHET et Sergio ORTIZ (Équipe Produits Naturels, Analyses et Synthèses (PNAS), CiTCoM UMR 8038 CNRS, Paris, Université de Paris, Faculté de Pharmacie), et le Pr Jean Luc WOLFENDER et le Dr Luis QUIROS (Phytochemistry and Bioactive Natural Products, School of Pharmaceutical Science, EPGL, University of Geneva, Geneva, Switzerland).

Les échantillons de plantes ont été collectés, extraits et fractionnés par l'équipe de Pharmacognosie du Dr R. GROUGNET ; la déréplication chimique des extraits et fractions végétales a été réalisée par l'équipe du Pr WOLFENDER et leur activité biologique sur *P. aeruginosa* ainsi que leur potentiel mécanisme d'action ont été évalués au sein du LMSM. Cette thèse a été financée par une bourse de l'Université du FUNAI (Nigeria) et de Campus France.

Dans ce contexte, les objectifs de cette thèse sont de :

(1) Déterminer les effets atténuateurs de la virulence de *P. aeruginosa* d'extraits, de fractions et de composés de plantes isolées du Chili ou d'Algérie (Programme de l'Union Européenne H2020-MSCA-RISE-2015 EXANDAS (Exploitation of aromatic plants' by-products for the development of novel cosmeceuticals and food supplements, Grant Agreement 691247).

(2) Décrypter le potentiel mécanisme d'action sous-jacent.

RÉSULTATS

PARTIE I. *Pistacia lentiscus*, UNE SOURCE DE COMPOSES ATTENUANT LA VIRULENCE DE *P. aeruginosa*

Pistacia lentiscus (*Anacardiaceae*) est également connu sous le nom d'arbre au mastic ou de Pistachier lentisque est un arbuste poussant dans les maquis et les garrigues des climats méditerranéens. Il est largement répandu dans certains pays d'Afrique du Nord (Maroc, Algérie, Tunisie, Libye, Égypte) et d'Europe méditerranéenne (Espagne, Portugal, France, Italie, Croatie, Albanie, Grèce) (Zohary, 1952; Al-saghir and Porter, 2012). C'est une plante de la famille des *Anacardiaceae* à feuillage persistant, qui donne des fruits d'abord rouges, puis noirs. Les applications médicinales des variétés méditerranéennes de *P. lentiscus* L. ont été signalées depuis des siècles. En Grèce, *Pistacia lentiscus* var. *chios* pousse dans l'île grecque de Chios et est principalement utilisé pour sa gomme appelée mastic (résine), pour la dentisterie et les problèmes gastro-intestinaux. En Afrique du Nord, plus particulièrement en Algérie, l'huile des fruits et des graines est utilisée pour le traitement des infections des voies respiratoires et comme onguents pour les douleurs articulaires, les brûlures et les ulcères (Bozorgi *et al.*, 2013; Rauf *et al.*, 2017). Malgré l'utilisation de cette plante dans ces régions en médecine traditionnelle, les propriétés médicinales de *Pistacia lentiscus* demeurent sous-explorées.

L'objectif de cette étude était de rechercher un potentiel effet anti-virulence sur *P. aeruginosa* d'extraits de fruits de *P. lentiscus* L., et son potentiel mécanisme moléculaire d'action. La récolte des fruits ainsi que l'extraction des fruits de *Pistacia lentiscus* L. a été réalisée par nos collaborateurs de l'équipe « Produits Naturels, Analyses et Synthèses » (PNAS, CiTCoM UMR 8038 CNRS, Paris, Université de Paris, Faculté de Pharmacie), entre septembre et octobre 2016 et 2018 dans la région

Nord de l'Algérie (Wilaya Jijel) dans le cadre du projet EXANDAS (Programme de l'Union Européenne H2020-MSCA-RISE-2015 EXANDAS (Exploitation of aromatic plants' by-products for the development of novel cosmeceuticals and food supplements, Grant Agreement 691247). Les fruits ont été extraits à l'aide de 4 solvants (cyclohexane, acétate d'éthyle, méthanol et eau) qui ont généré 4 extraits notés PLFE1-PLFE4. Cette étude rapporte que l'extrait cyclohexane de fruit de *Pistacia lentiscus* L. (PLFE1) atténue la virulence de *P. aeruginosa* en ciblant principalement la production de pyocyanine en interférant avec la production de molécules de communication de la famille des HAQs. Il apparaît que l'activité anti-virulence de l'extrait PLFE1 pourrait être associée à une altération de l'homéostasie membranaire par la modulation du facteur sigma à fonction extracytoplasmique SigX, impliqué dans la réponse au stress de l'enveloppe chez *P. aeruginosa*. Une analyse chimique approfondie de l'extrait PLFE1 couplée à une analyse biologique a permis d'identifier l'acide ginkgolique (C17: 1) et l'acide hydroginkgolique (C15: 0) en tant que principaux composés bioactifs pouvant interférer avec les membranes de *P. aeruginosa*.

L'ensemble de ces travaux a été consigné dans un article suivant :

Tahrioui A, Ortiz S, **Azuama OC**, Bouffartigues E, Benalia N, Tortuel D, Maillot O, Chemat S, Kritsanida M, Feuilleley M, Orange N, Michel S, Lesouhaitier O, Cornelis P, Grougnet R, Boutefnouchet S, Chevalier S. Membrane-Interactive Compounds From *Pistacia lentiscus* L. Thwart *Pseudomonas aeruginosa* Virulence. *Frontiers in Microbiology*. 2020 May 26;11:1068. doi: 10.3389/fmicb.2020.01068. eCollection 2020. PMID: 32528451

PARTIE II. ATTENUATION DE LA VIRULENCE *P. aeruginosa* PAR DES PLANTES MEDICINALES TRADITIONNELLES DU CHILI

I. Collection de plantes médicinales de la Communauté Taira (Atacama, Chili)

En raison de l'extrême aridité de la région d'Atacama, les espèces végétales habitantes se distinguent par leur capacité exceptionnelle à s'adapter à cet environnement extrême (Morong, 1891). Par conséquent, ces plantes peuvent posséder des composés phytochimiques uniques avec des effets anti-pathogènes. La collecte du matériel végétal a été réalisée par le Dr Sergio Ortiz au cours de son doctorat, sous la direction des Dr S. Boutefnouchet et R. Grougnet. Cette collecte a été effectuée en accord avec les représentants de la communauté Taira à deux reprises, du 11 au 13 septembre 2015 et du 9 au 11 de septembre 2016. Dix-huit plantes ont été récoltées, et déposées dans les collections de l'Université de Concepción (Concepción, Chili) et de l'équipe de Pharmacognosie de l'Université de Paris, Faculté des sciences pharmaceutiques et biologiques.

II. Criblage d'activités biologiques d'extraits de plantes contre *P. aeruginosa*

Le matériel végétal a été soumis à une extraction par un solvant sous pression, et une extraction par fluide super-critique a été réalisée selon les besoins par l'équipe de pharmacognosie (UMR 8038, Université de Paris). L'extraction par solvant sous pression a été utilisée en raison de son rendement d'extraction élevé : cette approche nécessite un minimum de temps et utilise peu de solvant. En utilisant pour solvant du méthanol et de l'acétate d'éthyl, 40 extraits (SE1-SE44) issus de 18 plantes appartenant à 8 familles ont été évalués pour leurs effets d'atténuation de la virulence de *P. aeruginosa*. Ces extraits ont été solubilisés dans du DMSO (1% final)

et testés à raison d'une gamme de concentrations comprises entre 0,8 et 100 µg/ml. La souche *P. aeruginosa* H103, qui est une souche prototrophe de la souche PAO1, issue du laboratoire de REW Hancock (Vancouver, Canada) ; a été utilisée au cours de cette étude, pour évaluer leur effet sur *P. aeruginosa* en termes de production de pyocyanine d'une part et de formation de biofilm d'autre part.

La pyocyanine est un facteur de virulence majeur chez *P. aeruginosa*, considérée comme un marqueur de l'activité du QS chez *P. aeruginosa*. Pour évaluer l'effet des extraits végétaux sur la production de pyocyanine, la souche H103 a été incubée en milieu LB à 37°C en absence (1% de DMSO) et en présence d'extraits (solubilisés dans 1% final de DMSO), sous agitation à 180 rpm pendant 24h en microplaque. La croissance bactérienne a été déterminée par lecture de l'absorbance à 580 nm. La quantification de la pyocyanine a été réalisée en ajoutant à la culture bactérienne, un volume de chloroforme pour en extraire la pyocyanine, avant de rajouter ½ volume de HCl 0,5 M à la fraction organique. L'absorbance de la fraction acide (couche rouge-rose) a été mesurée à 520 nm et a été rapportée à la croissance bactérienne (Tahrioui *et al.*, 2020; Essar *et al.*, 1990). En général, les extraits ont présenté divers effets sur la production de pyocyanine H103. Parmi ces 40 extraits, douze permettent d'atténuer la production de pyocyanine de *P. aeruginosa* H103 de 50% ou plus. Il s'agit des extraits acétate d'éthyl de *Lampaya medicinalis* Phil. (SE1, 54% de réduction), *Baccharis tola* Phil. ssp *tolu* (SE3, 55%), *Baccharis calliprinos* Griseb. (SE17, 55%), *Baccharis grisebachii* Hieron. (SE19, 55%), *Haplopappus rigidus* Phil. (SE21, 62%), *Senecio nutans* Sch. Bip (SE27, 62%), *Parastrephia teretiuscula* Cabrera (SE29, 60%), *Artemisa copa* Phil. Var. *copa* (SE39, 59% pour les feuilles) et d'*Azorella atacamensis* (SE5, 72%) ; et des extraits méthanol de *Haplopappus rigidus* Phil. (SE22, 61%), *Fabiana squamata* Phil. (SE12, 52%) et de *Polylepis tarapacana* Phil. (SE8, 55%). Les extraits d'acétate d'éthyl semblaient donc présenter une plus grande activité anti-pyocyanine par rapport à leurs homologues méthanol. De manière générale, cet effet

était dose-dépendant, le meilleur effet étant observé pour une concentration de 100 µg/ml d'extrait brut. De manière remarquable, sur ces 12 extraits actifs, 8 correspondent à des extraits de plantes appartenant à la famille des *Asteraceae*, 1 à la famille des *Verbanaceae*, 1 à la famille des *Apiaceae*, 1 à la famille des *Solanaceae* et 1 à la famille des *Rosaceae*. Ces données suggèrent qu'il pourrait y avoir dans les plantes de la famille des *Asteraceae*, un ou plusieurs composés d'intérêt en termes d'atténuation de virulence chez *P. aeruginosa*.

Pour évaluer l'effet des extraits sur la formation de biofilm chez *P. aeruginosa*, la souche H103 a été cultivée en condition statique à 37°C pendant 24h dans un milieu LB contenant du DMSO (1% final, condition témoin) ou du LB contenant l'extrait à différentes concentrations (entre 0,8 et 100 µg/ml) solubilisé dans du DMSO (1% final). Le biofilm formé a été coloré au crystal violet, avant d'être décroché par une solution d'éthanol à 70% et dosé à 405 nm par spectrophotométrie. Les extraits qui ont été initialement considérés comme ayant un effet atténuateur de la formation de biofilm, devaient réduire cette capacité d'au moins 50%. Aucun extrait ne rentrait dans cette catégorie selon ce critère. Cependant, certains extraits présentent une activité intéressante en termes de réduction de la formation du biofilm, mais uniquement à certaines concentrations. Il s'agit en particulier de l'extrait SE19 (réduction comprise entre 38 et 44 % entre 3,125 et 12,5 µg/ml) ; SE21 et SE22 (réduction comprise entre 22 et 29% ou entre 24 et 36%, respectivement aux mêmes concentrations que SE19) ; SE34 (réduction comprise entre 26 et 34 % entre 3,125 et 25µg/ml). De manière remarquable, de nombreux extraits augmentent la capacité de *P. aeruginosa* à former un biofilm, qui est, dans la plupart des cas, dose-dépendant. C'est le cas par exemple de l'extrait SE1, pour lequel il y a environ 3,5 fois plus de biofilm formé en présence de l'extrait comparativement à la condition témoin.

Parmi les 12 extraits potentiellement intéressants en termes d'atténuation de la production de pyocyanine, c.à.d. SE1, SE3, SE5, SE8, SE12, SE17, SE19, SE21, SE22, SE27, SE29 et SE39, les 5 extraits SE1, SE8, SE12, SE27 et SE29 favorisaient la formation de biofilm dans nos conditions, alors que les 7 extraits SE3, SE5, SE17, SE19, SE21, SE22, SE39 étaient relativement sans effet (<1,5 fois) sur la formation de biofilm.

Même si plusieurs extraits étaient potentiellement intéressants, notre choix s'est porté sur une étude plus approfondie des extraits de *A. atacamensis* (SE5), *Baccharis grisebachii* Hieron (SE19), *Haplopappus rigidus* Phil (SE21) et *Parastrephia teretiuscula* Cabrera (SE29). Ce choix a été fait sur la base de différents critères : l'effet sur la pyocyanine, l'effet sur la formation de biofilm, la quantité de matériel végétal disponible, et le temps nécessaire pour réaliser l'ensemble des essais. Ainsi, parmi ces 4 extraits choisis, 2 (SE19, SE21) présentaient à la fois une activité d'atténuation de la production de pyocyanine et dans une moindre mesure du biofilm (à certaines concentrations), 1 (SE5) réduisait la production de pyocyanine sans affecter la formation de biofilm même à une concentration de 100µg/ml, et 1 réduisait la pyocyanine mais augmentait la formation de biofilm d'un facteur d'environ 2,5 fois (SE29). Dans ce dernier cas, la réduction de pyocyanine étant de l'ordre de 60%, nous avons envisagé le fait que des composés différents pouvaient être impliqués dans ces 2 effets.

Nous avons ainsi poursuivi notre étude sur *A. atacamensis* (SE5), objet du Chapitre III de ce manuscrit, puis sur 3 membres de la famille des *Asteraceae* (Chapitre IV), à savoir *Parastrephia teretiuscula* Cabrera (SE29), *Baccharis grisebachii* Hieron (SE19), *Haplopappus rigidus* Phil (SE21).

III. *Azorella atacamensis* comme source de composés actifs contre *P. aeruginosa*

Les espèces d'*Azorella* sont communes en Amérique du Sud, dans le sud-est de l'Australie, en Nouvelle-Zélande et dans les îles de l'océan Austral et appartiennent à la famille des Apiaceae. Les espèces de cette famille sont des arbustes formant un tapis jaune-vert d'environ 15 cm. Parmi les espèces les plus courantes, on retrouve *Azorella compacta* Phil. également connue sous le nom de «yareta» en langue quechua, et *Azorella atacamensis* G.M.Plunkett & A.N.Nicolas (syn. *Mulinum crasiifolium* Phil.) communément connue sous le nom de Chuquicandia ou Chuquican par la communauté Taira localisée dans le désert d'Atacama (Chili). Utilisées en médecine traditionnelle, les fleurs, graines et résines de ces plantes sont généralement prises sous forme d'infusions dans le traitement des troubles urinaires, digestifs, bronchiques et respiratoires (Ortiz *et al.*, 2019). Les principaux composés chimiques isolés à partir de diverses espèces comprennent des diterpénoïdes, monoterpènes, acides mulinéique et mulinologique qui pourraient exercer des activités anti-plasmodiales, anti-inflammatoires et antibactériennes (Bórquez *et al.*, 2014). Par exemple, les huiles essentielles d'*Azorella trifurcata* exercent une activité antibactérienne contre les espèces *Pseudomonas* et *Staphylococcus coagulase négative* à des CMI de 500 µg/ml et 1000 µg/ml respectivement (Lopez *et al.*, 2018). L'effet antibactérien de l'extrait d'acétate d'éthyle d'*Azorella atacamensis* contre *Bacillus cereus* et *Corynebacterium minutissimum* à des concentrations de 60 µg/ml et 40 µg/ml a également été rapporté dans une étude précédente (Ortiz *et al.*, 2019).

Lors du criblage de 40 extraits de plantes obtenus à partir de 18 plantes médicinales (Communauté Taira Atacama) pour leurs effets atténuateurs sur la production de biofilm et de pyocyanine, l'extrait d'acétate d'éthyle d'*Azorella*

atacamensis SE5), a présenté le plus fort effet en termes de réduction de pyocyanine, sans affecter la formation de biofilm. Les travaux présentés ici visaient à i) préciser l'effet anti-virulence des extraits d'acétate d'éthyle d'*Azorella atacamensis* (notés AaE) contre la souche *Pseudomonas aeruginosa* H103 ; ii) identifier des fractions actives, iii) identifier des composés bioactifs, et iv) décrypter un potentiel mécanisme moléculaire sous-jacent.

Nous avons ainsi montré que l'extrait d'*Azorella atacamensis* (AaE) a conféré une protection significative contre *P. aeruginosa* sur un modèle cellulaire (cellules pulmonaires humaines A549) et sur un organisme complet, *Caenorhabditis elegans*. La production de plusieurs facteurs de virulence et de molécules du QS a diminué lors de l'exposition à cet extrait sans affecter la croissance de *P. aeruginosa*. Le profilage phytochimique de l'extrait AaE et de ses fractions, obtenu en combinant une déplication chimique et des approches de construction in silico de réseau moléculaire, a permis l'identification potentielle de 14 métabolites portant un squelette de type mulinane. De manière remarquable, ce groupe de diterpénoïdes semble être responsable de l'atténuation de la virulence de *P. aeruginosa*, probablement par perturbation de l'homéostasie membranaire bactérienne. L'augmentation significative de la rigidité membranaire de *P. aeruginosa*, observée suite au traitement par ces extraits, fractions ou composés, semble de plus être modulée par SigX, un facteur sigma de fonction extracytoplasmique impliqué dans l'homéostasie membranaire ainsi que la virulence de *P. aeruginosa*.

Ces travaux ont été réalisés en collaboration avec l'Équipe « Produits Naturels, Analyses et Synthèses » (PNAS), CiTCoM UMR 8038 CNRS, Paris, Université de Paris, Faculté de Pharmacie, (collection, extraction, fractionnement et purification de composés), et le laboratoire "Phytochemistry and Bioactive Natural Products", School of Pharmaceutical Science, EPGL, University de Geneve, Suisse

(déréplication et construction de réseaux moléculaires). Ils ont été soumis pour publication le 10 novembre 2020 dans le journal « Biomolécules », dans une issue spéciale intitulée : « Pharmacology of Medicinal Plants » (**ID-biomolécules-1014572**)

*IV. Attenuation de la virulence de *Pseudomonas aeruginosa* par des extraits de *Parastrephia teretiuscula* Cabrera, *Baccharis grisebachii* Hieron et *Haplopappus rigidus* Phil.*

Parastrephia teretiuscula Cabrera est un petit arbuste à feuilles persistantes de 5 à 20 cm de hauteur que l'on trouve largement répandu au Pérou et dans les Andes, à des altitudes élevées (3000 à 4200 m). Les parties aériennes de diverses espèces sont utilisées traditionnellement pour traiter les maux de dents, les fractures osseuses et les ecchymoses. Différents composés, (isofraxidine, flavonoïdes, esters d'E-caffeoyl ou E-cinnamoyl, scopolétine, *p*-coumaroyl-oxytrémétone, ombelliférone, composés phénylpropanoïdes et phénoliques) ont été décrits pour exercer des effets antimicrobiens (Dalmeida *et al.*, 2012).

Les espèces de *Baccharis* sont des arbustes de 30 cm à 2 m de hauteur, poussant principalement dans l'Altiplano, au nord-ouest de l'Argentine et au nord du Chili. Les parties aériennes de ces espèces sont utilisées en médecine traditionnelle en infusions ou sous forme de cataplasme dans la cadre d'un traitement de troubles digestifs, brûlures, problèmes osseux ou plaies dans la communauté Atacama et en Argentine (Indena (Firm) *et al.*, 1996).

Les espèces de *Haplopappus*, également connues sous le nom de Bailahuén, sont des arbustes que l'on trouve principalement dans les Andes au Chili. Les feuilles et

les tiges sont couramment utilisées en médecine traditionnelle dans le traitement des maladies hépatiques, gastro-intestinales et cholérétiques (Schrickel et Bittner, 2001). Les métabolites secondaires isolés des exsudats résineux de *H. rigidus* sont principalement des monoterpènes, terpénoïdes, flavonoïdes ou encore la coumarine, par exemple. Ces composés ont un effet anti-oxydant (González *et al.*, 2012; Torres *et al.*, 2004, 2006).

Lors du criblage de 40 extraits de plantes médicinales, nous avons montré que :

- l'extrait d'acétate d'éthyle de *Parastrephia teretiuscula* Cabrera SE29 induisait une réduction de 60% de la pyocyanine présente dans un surnageant de culture de *P. aeruginosa* H103 à une concentration de 100 µg/ml. Bien que cet extrait augmentait la formation du biofilm d'un facteur d'environ 2,5 fois à cette même concentration, nous l'avons sélectionné pour une étude plus approfondie de par son effet important sur la production de pyocyanine. De plus, nous avons émis l'hypothèse que des composés différents pouvaient être impliqués dans ces 2 phénotypes, ce qui permettait potentiellement d'envisager de les séparer en vue d'une application future.

- l'extrait d'acétate d'éthyle de *Baccharis grisebachii* Hieron SE19 induisait une réduction de 55% de la pyocyanine présente dans un surnageant de culture de *P. aeruginosa* H103, sans affecter la formation de biofilm à une concentration de 100µg/ml. Cependant, à une concentration plus faible, comprise entre 1,625 et 25 µg/ml, cet extrait permettait de réduire la capacité de *P. aeruginosa* H103 à former un biofilm, tout en conservant un effet de réduction de la production de pyocyanine, bien que plus limité.

- l'extrait d'acétate d'éthyle de *H. rigidus* SE21 induisait une réduction de 62% de la pyocyanine présente dans un surnageant de culture de *P. aeruginosa* H103, sans affecter la formation de biofilm à une concentration de 100µg/ml. Cependant, à une concentration plus faible, comprise entre 0,8 et 12,5 µg/ml, cet extrait permettait de

réduire la capacité de *P. aeruginosa* H103 à former un biofilm (entre 2à et 29%), tout en conservant un effet de réduction de la production de pyocyanine, bien que plus limité.

Nous avons donc sélectionné ces 3 extraits pour une étude plus approfondie du potentiel effet atténuateur de virulence et des mécanismes potentiels impliqués. Les travaux présentés dans ce chapitre visaient à préciser l'effet anti-virulence des extraits d'acétate d'éthyle de *Parastrephia teretiuscula* Cabrera (notés PtE), *Baccharis grisebachii* Hieron (notés BgE) et *H. rigidus* (notés HrE), contre la souche *P. aeruginosa* H103 ; identifier des fractions actives, et décrypter un potentiel mécanisme moléculaire sous-jacent.

Nous avons montré que ces 3 extraits provoquent une diminution de la cytotoxicité contre les cellules pulmonaires humaines sans affecter la croissance de *P. aeruginosa* ni montrer de toxicité pour les cellules pulmonaires. De même, une diminution de la production des facteurs de virulence pyocyanine et élastase a été observée après exposition à des extraits de plantes. Le système de quorum sensing de type HAQ (PQS) est affecté par les extraits de plantes, et une rigidification de la membrane bactérienne a été observée après exposition aux extraits. Des fractions actives ont été identifiées. Les extraits et fractions sont actuellement en cours de dérégulation par nos collègues de Genève (Pr JL Wolfender et L. Queiroz), et la purification de composés potentiellement actifs est en cours par nos collègues du laboratoire de Pharmacognosie de l'Université de Paris Descartes (Dr S. Boutefnouchet et Dr R. Grougnet) dans le but de compléter un manuscrit en cours de rédaction.

DISCUSSION

P. aeruginosa a été classée récemment par l'OMS parmi les agents pathogènes à l'origine des plus grandes menaces de maladies infectieuses auxquels sont/seront confrontés les médecins d'aujourd'hui. En effet, l'absence d'outils thérapeutiques face à l'émergence de souches résistantes à certains antibiotiques ou même Pan-Drug-Résistantes (résistantes à tous les antibiotiques) est devenue une réalité et la recherche de nouveaux agents antibactériens est donc un enjeu de santé publique, comme le démontre la création du programme prioritaire de lutte contre la résistance aux antibiotiques décidée par le gouvernement français fin 2018, programme coordonné par l'INSERM pour le compte de l'ensemble de la communauté scientifique nationale.

Les travaux réalisés dans le cadre de cette thèse ont permis de mettre en évidence plusieurs extraits, fractions voire composés ayant une activité intéressante en termes d'atténuation de la virulence de *P. aeruginosa*. De manière remarquable, cet effet était plus important que ceux rapportés dans les études précédentes pour d'autres plantes. Par exemple, la fraction acétate d'éthyle de *Acacia hockii*, utilisée en médecine traditionnelle au Bénin, conduit à une diminution de la production de pyocyanine de 45,5%, lorsqu'elle est utilisée à des concentrations de 100 µg / ml (Tchatchedre *et al.*, 2019). De même, l'extrait éthanolique de *Plantago asiatica* L, couramment utilisé en médecine traditionnelle chinoise, a montré une activité atténuatrice de la production de pyocyanine à des concentrations de 8 à 16 µg / mL (Li *et al.*, 2019). Dans l'ensemble, ces données ont montré que ces extraits présentaient un potentiel significatif en tant qu'atténuateurs de la production de pyocyanine chez *P. aeruginosa*, et nous avons choisi de nous concentrer sur ces extraits pour des recherches plus approfondies. La pyocyanine possède de nombreux effets toxiques sur les cellules de mammifères, probablement par génération d'un stress oxydatif

(Hall *et al.*, 2016; Ray *et al.*, 2015). En accord avec ce phénotype, cette diminution de la production de pyocyanine a pu être reliée à une diminution de la cytotoxicité sur cellules épithéliales pulmonaires et de la virulence globale sur un organisme entier simple, *C. elegans*. Cependant, cette virulence et cytotoxicité sont des phénotypes complexes et multifactoriels (Mahajan-Miklos *et al.*, 1999; Gallagher et Manoil, 2001; Engel et Balachandran, 2009; Cezairliyan *et al.*, 2013), il est possible que d'autres facteurs de virulence tels que le cyanure d'hydrogène (HCN) ou encore des effecteurs du système de sécrétion de type III puissent être également affectés par les actifs végétaux. Il serait donc intéressant de doser la production d'autres facteurs de virulence. La production de la plupart de ces facteurs de virulence étant sous le contrôle du QS, nous avons observé que ces extraits, fractions ou composés d'origine végétale avaient un effet atténuateur de la production de molécules de communication du QS, à savoir le système HAQ dans tous nos travaux, et également du système AHL dans le cas de *Pistachia lenticus* L. Plusieurs études ont rapporté la capacité de certains composés phytochimiques à atténuer le QS et à réguler ainsi l'expression de facteurs de virulence. Par exemple, l'extrait d'éthanol et les fractions d'acétate d'éthyle de *Centella asiatica* L. atténuent la production des AHLs, en plus de la pyocyanine, de l'activité élastase chez *P. aeruginosa* PAO1 (Vasavi *et al.*, 2016). Dans l'ensemble, nos données suggèrent que les extraits de plantes pourraient interférer avec une partie de la production d'autoinducteurs QS. Au cours de ces dernières années, un grand nombre d'études ont rapporté des produits naturels ciblant des facteurs de virulence (Silva *et al.*, 2016). Plusieurs composés d'origine végétale comme des alcaloïdes, des organosulfurés, des coumarines, des flavonoïdes, des acides phénoliques, des phénylpropanoïdes, ou encore des terpénoïdes chimiques se sont révélés actifs contre la production de pyocyanine (Silva *et al.*, 2016). Cependant les mécanismes moléculaires d'action sous-jacents spécifiques, n'ont que très peu été abordés. Les extraits choisis dans cette étude correspondaient à des fractions acétate d'éthyle des différentes espèces végétales,

composées donc au moins partiellement de molécules partiellement apolaires. Nous avons donc émis l'hypothèse que ces composés pouvaient perturber au moins en partie la membrane externe de *P. aeruginosa*, membrane localisée à l'interface entre la bactérie et l'environnement (Tahrioui *et al.*, 2020; Flechard *et al.*, 2018; Bouffartigues *et al.* 2020). Nous avons donc évalué l'homéostasie membranaire de *P. aeruginosa* en réponse à chacun des extraits, fractions ou composés, par mesure de l'anisotropie de fluorescence et évaluation de sa rigidité ou fluidité. L'exposition des extraits d'acétate d'éthyle de *B. grisebachii*, *H. rigidus* Phil et *P. teretiuscula* à *P. aeruginosa* H103 a conduit à une rigidification de la membrane de 20,06%, 27,33% et 23,5% respectivement, alors que l'extrait d'acétate d'éthyle d'*A. atacamensis* et l'extrait cyclohexane de *Pistachia lentiscus* a augmenté la rigidité de la membrane de *P. aeruginosa* de 38,25% et 20% respectivement. Ces résultats impliquent des changements physiologiques et une adaptation de la membrane bactérienne en réponse à l'exposition des extraits végétaux. Enfin, nous avons montré que ce phénotype pourrait être lié au moins partiellement au facteur sigma à fonction extracytoplasmique SigX, qui contrôle l'homéostasie membranaire chez *P. aeruginosa* (Boechat *et al.*, 2013; Duchesne *et al.*, 2013 ; Fléchard *et al.*, 2018; Bouffartigues *et al.*, 2020; Tahrioui *et al.*, 2020; Chevalier *et al.*, 2019).

En raison de la complexité des extraits et fractions d'origine végétale, une stratégie basée sur leur dé-réplication spectrale a été choisie (Wolfender *et al.*, 2019). Pour ce faire, les spectres des extraits et fractions ont été comparés à une base de données de spectres de métabolites d'origine végétale connus. Ces données ont été associées aux données biologiques afin de proposer des molécules candidates ou familles de molécules dotées de l'activité biologique. Cette stratégie a été appliquée dans le cas des extraits et fractions d'*A. atacamensis*, et a permis l'identification putative de quatorze métabolites portant un squelette de type mulinane. De manière remarquable, ce groupe unique de diterpénoïdes semble être responsable de

l'interférence avec la production des facteurs de virulence ainsi que de la perturbation de l'homéostasie membranaire de *P. aeruginosa*. La purification ainsi que l'identification par RMN de ces diterpénoïdes bioactifs de type mulinane, devra être réalisée et leur activité biologique devra être testée sur *P. aeruginosa* H103 ainsi que sur des souches d'origine clinique. Ces composés devront également être testés en synergie avec les antibiotiques conventionnels. Ce type de stratégie a également été mise en place pour les autres extraits et fractions de plantes chiliennes, et devraient pouvoir aboutir sur l'identification de nouveaux composés à activité atténuatrice de virulence chez *P. aeruginosa*. Enfin, des analyses de type omic (RNA-seq, protéomique) permettraient d'élucider les mécanismes moléculaires mis en jeu par ces composés et de découvrir de potentielles nouvelles cibles thérapeutiques.

TRAVAUX ADDITIONNELS

- Tortuel D, Tahrioui A, Rodrigues S, Cambrone M, Boukerb AM, Maillot O, Verdon J, Bere E, Nusser M, Brenner-Weiss G, David A, **Azuama OC**, Feuilloley MGJ, Orange N, Lesouhaitier O, Cornelis P, Chevalier S, Bouffartigues E. 2020. Activation of the Cell Wall Stress Response in *Pseudomonas aeruginosa* Infected by a Pf4 Phage Variant. *Microorganisms*. 8:E1700. doi: 10.3390/microorganisms8111700.

- Fléchar M, Duchesne R, Tahrioui A, Bouffartigues E, Depayras S, Hardouin J, Lagy C, Maillot O, Tortuel D, **Azuama OC**, Clamens T, Duclairoir-Poc C, Catel-Ferreira M, Gicquel G, Feuilloley MGJ, Lesouhaitier O, Heipieper HJ, Groleau MC, Déziel É, Cornelis P, Chevalier S. The absence of SigX results in impaired carbon metabolism and membrane fluidity in *Pseudomonas aeruginosa*. *Scientific Reports*. 2018 Nov 21;8(1):17212. doi: 10.1038/s41598-018-35503-3.

- Bouffartigues E, Si Hadj Mohand I, Maillot O, Tortuel D, Omnes J, David A, Tahrioui A, Duchesne R, **Azuama OC**, Connil N, Lesouhaitier O, Bazire A, Orange N, Feuilleley MGJ, Dufour A, Cornelis P, Chevalier S. (2020) The temperature-regulation of *Pseudomonas aeruginosa* *cmaX-cfrX-cmpX* operon reveals an intriguing molecular network involving the sigma factors AlgU and SigX. *Frontiers in Microbiology*. 11:579495. doi: 10.3389/fmicb.2020.579495

CONCLUSIONS

A ce jour, les travaux réalisés au cours de ma thèse sont consignés dans une revue en cours de préparation (1^{er} auteur), un article publié (3^{ème} auteur), un article soumis (1^{er} auteur), un article en cours de préparation (1^{er} auteur). J'ai également contribué à 3 articles publiés en tant que coauteur (9^{ème}, 10^{ème}, 12^{ème} auteur).

Recherche de nouveaux actifs d'origine végétale contre le pathogène opportuniste de l'Homme *Pseudomonas aeruginosa*

La résistance aux antimicrobiens est l'un des défis majeurs du XXI^{ème} siècle. *Pseudomonas aeruginosa* est inscrit sur la liste des organismes pathogènes qui deviennent résistants aux antibiotiques conventionnels. De nouvelles stratégies visant à atténuer la virulence sans perturber la croissance et la viabilité bactériennes, également connues sous le nom de stratégie anti-virulence, sont développées. Les plantes sont connues pour produire de nombreux métabolites secondaires. Des extraits de fruits de *Pistacia lentiscus* originaires d'Algérie et de 40 extraits de plantes originaires du Nord-Chili ont été criblés pour leur capacité à atténuer la production de la pyocyanine, un facteur de virulence majeur de *P. aeruginosa*, dans le but d'évaluer leur potentiel effet anti-virulence. Les extraits sélectionnés (*Pistacia lentiscus*, *Azorella atacamensis*, *Baccharis grisebachii*, *Haplopappus rigidus* et *Parastrephia terestiucula*), ont été fractionnés et l'ensemble de ces extraits et fractions a montré une atténuation de la production d'autres facteurs de virulence (élastase, rhamnolipides), qui a pu être attribuée, au moins partiellement à une diminution de la communication bactérienne via le mécanisme du quorum sensing. Ces extraits et fractions altèrent également la fluidité membranaire de *P. aeruginosa*. Cet effet anti-virulence a été validé dans un modèle d'infection cellulaire, et sur le nématode *Caenorhabditis elegans*. Dans toutes ces conditions, la croissance de *P. aeruginosa* n'a pas été affectée. Un profilage chimique des extraits et fractions de *P. lentiscus* et d'*A. atacamensis* a révélé la présence d'acide ginkgolique et de diterpénoïdes de type azorellane/mulinane comme potentiels composés bioactifs. De futures études visent à identifier les composés bioactifs sur *P. aeruginosa* H103, ainsi que sur un panel de souches cliniques, et à évaluer un potentiel effet potentialisateur de l'activité des antibiotiques. Ces travaux visent in fine à proposer ces composés d'origine végétale comme adjuvants dans le traitement des infections à *P. aeruginosa*.

Mots clés: *Pseudomonas aeruginosa*, infections, anti-virulence, extraits et composés végétaux

Searching for new bioactive compounds to tackle the opportunistic human pathogen *Pseudomonas aeruginosa*

Antimicrobial resistance has become a great challenge in therapeutic medicine so much so that the World health organization forecasts the possibility of a post-antibiotic era where minor injuries may lead to mortality. *Pseudomonas aeruginosa* is among the list of organisms that are highly resistant to conventional antibiotics, partly due to its broad genome, which facilitates the elaboration of virulence determinants and rapid adaptation to various environments, in addition to its inherent resistance mechanisms. In view of this, alternative measures of controlling microbial virulence activities using novel approaches that do not disturb its growth and viability, also known as anti-virulence strategy, are gaining wider attention. Since plants are repositories of several metabolites with chemical defense system against environmental pathogens, through ethnobotanical led studies, the effect of *Pistacia lentiscus* fruit extracts originating from Algeria and forty plant extracts originating from North-Chile were biologically and chemically evaluated with the aim of deciphering their anti-virulence effects against *P. aeruginosa*. Furthermore, this study tried to gain more insight into the bioactive compounds and possible mechanism of action. From the results obtained, selected plant extracts attenuated *P. aeruginosa* mainly pyocyanin activity and /or elastase and rhamnolipids virulence production which appears to be associated with the inhibition of quorum sensing activities and the alteration in membrane activities. The anti-virulence effect of the selected extracts (*P. lentiscus*, *Azorella atacamensis*, *Baccharis grisebachii*, *Haplopappus rigidus* and *Parastrephia terestiucula*) were also validated in biological models of infections where they mediated the toxicity of *P. aeruginosa* towards A549 human monolayer cells and/or *Caenorhabditis elegans* nematode. Interestingly, growth of the pathogen was not affected. Further chemical profiling of *P. Lentiscus*, and *A. atacamensis* extracts revealed the presence of ginkgolic acid and azorellane/mulinane diterpenoids as the putative bioactive compounds. Future studies intend to explore these extracts and their derived compounds on the potentiation of antibiotic activity in a panel of clinical strains. In general, this study sets the pace for the possible use of these plant extracts as adjuvants in treatment of *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, infections, anti-virulence, plant extracts and compounds