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Chapter

Pyoverdine as an Important Virulence Factor in *Pseudomonas aeruginosa* Antibiotic Resistance

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Abstract

The World Health Organization has designated *P. aeruginosa* as a priority one pathogen due to the rise of multidrug-resistant (MDR) strains. It is a common opportunistic pathogen among humans. Nosocomial pneumonia, hospital-acquired urinary tract infection, and surgical wound infections are all caused by it. *P. aeruginosa* causes significant rates of disease and death in immunocompromised people such as those who have had a bone marrow transplant, have cystic fibrosis, have had burns, or have AIDS. *P. aeruginosa's* ability to cause such a wide range of infections is owing to its arsenal of virulence factors, which includes pyoverdine molecules, which are responsible for MDR strains. Pyoverdines are nonribosomal short peptides that are essential for bacterial pathogenicity because they serve as a signal molecule for the development of other virulence factors and contribute to antibiotic resistance. Because they are formed under iron-limiting conditions in the host environment, siderophores are required for iron uptake in the host.

Keywords: pyoverdine, antibiotic resistance, *Pseudomonas aeruginosa*, *virulent factor*

1. Introduction

1.1 The genus *P. aeruginosa* and its medical importance

The taxonomy is as follows: Kingdom Monera, phylum Proteobacteria, class gamma subdivision, order *Pseudomonadaceae*, genus *Pseudomonas*, and species *P. aeruginosa*, and it was first described by Gessard in 1882 [1]. *Pseudomonas* is derived from two Greek words: Pseudo, which means "false," and monas, which means "single unit," while aeruginosa, which means "greenish-blue," comes from the Latin aerg, which means "rusted copper" [2]. It is a gram-negative, straight, or slightly curved bacillus with a length of 1–5 m and a width of 0.5–1.0 m. The presence of a polar flagellum, which is made up of a complex protein structure that allows for mobility in liquid environments and reaction to chemical stimuli, makes *P. aeruginosa* mobile. It can also bind to cell membranes thanks to this property. It has small filaments called pili, which are located on the outside. These structures are used to

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move in semisolid media and, like the flagellum, adhere to surfaces. Its morphology is heterogeneous, its colonies are generally large, flattened, smooth, or with serrated edges and may show a metallic sheen.

In relation to its metabolism, it is aerobic although it can develop under anaerobic conditions using nitrate as the terminal electron acceptor. It is a ubiquitous organism in the environment and also, it can colonize multiple niches and utilize many environmental compounds as energy sources. It is found mainly in water, soil, swamps, coastal marine habitats, as well as in plant and animal tissues as well as in hospitals. *P. aeruginosa* is characterized by producing a variety of pigments, such as pyocyanin (blue-green in color), pyoverdine (PVD) (yellowish green fluorescent pigment), and pyorubin (red). P. aeruginosa forms biofilms on moist surfaces such as rocks and soils [3–6].

This bacterium is an extremely important pathogen, since it is responsible for a high percentage of nosocomial infections in patients confined in health centers. As an opportunistic human pathogen, it is responsible for infection in immunocompromised patients such as cystic fibrosis, diabetes, cancer, severely burn patients, advanced HIV infections (acquired immunodeficiency syndrome, AIDS), bone marrow transplants, surgical wound infections, and catheterized patients, and this is as a consequence of its resistance to antibiotics and disinfectants that kill other environmental bacteria [7]. A broad range of cell-associated and external factors influence multidrug resistance and thus bacterial pathogenicity. In the colonization, survival, and invasion of tissues of bacteria, virulence factors play a crucial pathogenic function. The pili are responsible for adhesion to the epithelium. Exoenzyme S and other adhesins help epithelial cells stick together. Tissue necrosis is caused by the exotoxin A. Phospholipase C is a hemolysin that is thermolabile. Exoenzyme S's pathogenic involvement is due to its disruption of normal cytoskeletal organization, degradation of immunoglobulin G and A, depolymerization of actin filaments, and contribution to macrophage resistance. At least four proteases produced by *P. aeruginosa* cause bleeding and tissue necrosis. Siderophores (pyoverdin and pyochelin), which allow bacteria to proliferate in the absence of ferrous ions, are involved in chronic infection [4, 8]. The bacteria in strains recovered from cystic fibrosis patients have an alginate pseudocapsule that protects them against phagocytosis, dehydration, and antibiotics. It also enhances biofilm formation by adhering to epithelial cells. Most of these virulence factors are controlled by two different types of regulation systems: the two-component transcriptional regulatory system and the quorum sensing system. These two pathways are required for the microorganism's survival and multiplication in the host (**Figure 1**).

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Prior to 1966, no comprehensive investigation of the aerobic pseudomonads taxonomy had been conducted. It is the work of Stanier and collaborators [10], in which physiological and biochemical features were used to demonstrate the taxonomic basis for the species identification. The genus was amended in 1984 by Palleroni, and five groups were established based on the results of DNA–DNA hybridization and rRNA–DNA hybridization. All five groups were later identified as belonging to the class Proteobacteria, and members of the genus *Pseudomonas* "sensu stricto" were found to belong to the subclass Gammaproteobacteria's RNA–DNA group I. The rRNAI group is also subdivided into fluorescent and nonfluorescent bacterium [11].

The genome size of *P. aeruginosa*, which ranges between 5.5 and 7 megabytes, is the largest in bacteria and has the greatest environmental adaptability. The PAO1 strain is the

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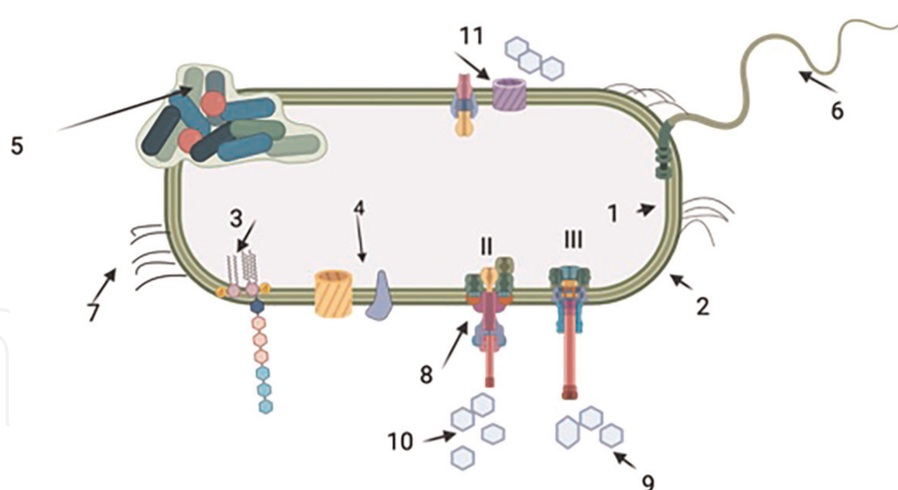

Figure 1.
P. aeruginosa *main virulent factors. Outer membrana(1), inner membrane (2), lipopolysaccharide (3), outermembrane proteins, porins and lipoproteins (4), biofilm (5), flagellum (6), pili (7), secretion systems (8), exotoxins (9), proteases, lipases, elastases, pyocianin (10), pyoverdine siderophore as an iron uptake system [9]. Created with BioRender.com.*

key reference strain for genetic and functional investigations on *P. aeruginosa*. It was first isolated from a wound of an Australian patient in the 1950s. The PAO1 genome is a 6.264-Mbp circular chromosome that encodes 5700 genes, including 5584 projected open read frames (ORFs), and was fully sequenced in 2000. As a result, it has the highest proportion of regulatory genes of any bacterial genome, as well as many genes involved in catabolism, transport, and efflux of organic compounds, as well as four potential chemotaxis systems. The size and complexity of the *P. aeruginosa* genome are thought to be an evolutionary adaptation that allows it to thrive in a variety of habitats while also resisting the effects of antimicrobial drugs. Knowledge of the complete genome sequence and encoded processes provides a wealth of information for the discovery and exploitation of new antibiotic targets, and the hope of developing more effective strategies to treat life-threatening opportunistic infections caused by *P. aeruginosa* in humans [12].

Studies on the P. aeruginosa transcriptome became possible after the genome was completed [13]. Understanding the lifestyle and pathogenicity of *P. aeruginosa* requires gene expression profiling. Following the introduction of next-generation sequencing systems, high-throughput sequencing of cDNA fragments became an alternative to microarray hybridization. The widespread use of this method, dubbed RNA-Seq or RNA-seq, resulted in an exponential rise in the number of whole transcriptome investigations published in the literature [14]. The transcriptomes of *P. aeruginosaclone C* were studied with this technology, and it was discovered that rRNA molecules accounted for 50 to 90 percent of the sense RNAs, followed by mRNA transcripts and noncoding RNA in comparable quantities. Uncharged tRNAs and 29 yet-undescribed antisense tRNAs were found in similar numbers and identified yet-undescribed RNA molecules. The identification of sense-antisense pairs of transfer-messenger RNA (tmRNA), tRNAs, and mRNAs using this RNA seq method implies a new level of gene regulation in bacteria [15].

Although the 16S rRNA gene is the basic tool of the current bacterial classification system, it is known that closely related bacterial species cannot be differentiated based on this gene. Therefore, in the last 10 years, other gene sequences have been used as phylogenetic molecular markers in taxonomic studies, such as atpD, gyrB, rpoB, recA, and rpoD [16]. Mulet and collaborators have shown that analysis of the sequences of

four housekeeping genes (16S rRNA, gyrB, rpoB, and rpoD) in all known species of the genus clarified the phylogeny and greatly facilitated the identification of new strains. Multilocus sequence typing (MLST) of the four housekeeping genes is reliable for species delineation and strain identification in Pseudomonas [17]. MLST is enhancing our understanding of the general genome organization of *P. aeruginosa* strains and species, and it is the standard method used for epidemiological surveys on *P. aeruginosa* outbreaks worldwide [18, 19].

2. Iron role in metabolism

Iron is a micronutrient found in almost all living organisms and is an essential component of nearly all of them [20]. It can be present in both reduced (Fe^{2+}) and oxidized (Fe^{3+}) forms in cells, making it simple to insert into an enzyme's catalytic site and serve as an electron carrier in many redox-sensing proteins. Iron forms part of a larger cofactor such as Fe-S clusters and heme, the former is involved in diverse biological processes, including metabolite biosynthesis, DNA replication, RNA modification, gene expression, photosynthesis, and respiration, and the latter is required for cytochrome biogenesis and the transport and storage of oxygen in vertebrates. Iron is associated with oxidative stress. In the presence of oxygen, the ferrous ion is unstable, forming ferric ions and reactive oxygen species (ROS), which can damage biological macromolecules and cause cell death. This process is illustrated by the Fenton Reaction [21].

Fenton Reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Even though iron is the fourth most prevalent element in the Earth's crust, only its ferrous form is soluble in water, whereas ferric iron has very low solubility and forms insoluble precipitates hydroxides at neutral pH with solubilities of 10–9 to 10–10 M (i.e. 56 ng/L) [22]. Because the concentration is too low to maintain life, all organisms have evolved unique mechanisms to solubilize iron. After absorption of iron in the ferrous form by the protein ferroportin in the duodenal mucosa, animals absorb it from the meal. The iron is then transported to the glycoprotein transferrin, where it becomes ferric, and is then stored in ferritin as a polymeric ferric complex. This is utilized to feed iron to various apoproteins for them to produce various iron-containing proteins as well as to provide the iron required for erythrocyte development and hemoglobin synthesis [22, 23].

P. aeruginosa can acquire iron from different sources of its host organism, among these sources are (**Figure 2**):

- Transferrin and the related protein lactoferrin:Milk and other extracellutlar fluids contain it (saliva, tears, and nasal mucus). (Transferrin (Tf) is an iron carrier glycoprotein (Fe 3+), synthesized and metabolized mainly in hepatocytes. It is made up of a single polypeptide chain of 679 amino acids with a molar mass of 79,500 g/mol. Each transferrin molecule consists of two lobes with a similar internal structure and is independent for Fe 3+ fixation; the N-terminal lobe contains residues 1–336 and the C-terminal residues 336–679. Each lobe in turn is folded, forming two domains. This conformation of the molecule allows the firm, although reversible, union of Fe.

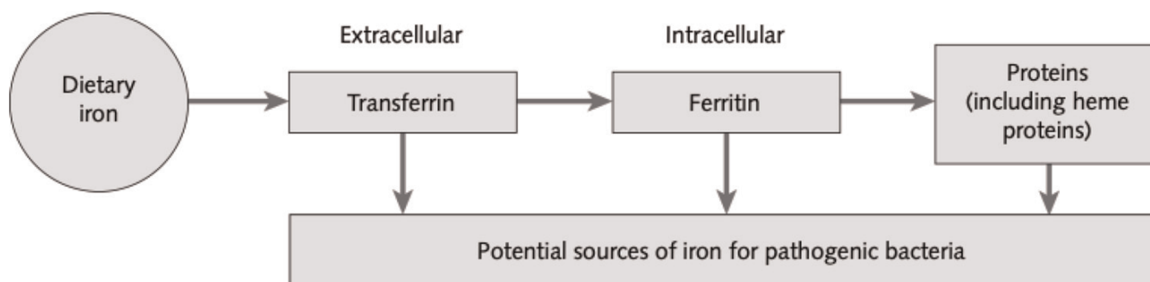

Figure 2.
Outline of the principal iron sources that may be accessed by bacterial pathogens source [23].

- Ferritin: Ferritin is the intracellular protein responsible for the storage and release of iron. Ferritin can store up to 4500 iron atoms as a ferrihydrite mineral in a protein shell and releases these iron atoms when needed by the cell. The ferritin protein coat consists of 24 protein subunits of 2 types, the H subunit and the L subunit.

- Fe-containing proteins such as heme proteins: In these proteins, iron is in its ferrous form and, as such, can be used as an appropriate ligand in which O2 can bind to be transported around the body as oxyhemoglobin.

Pathogens obtain iron from their hosts by three methods that are engaged when the bacterium is in an iron-deficient environment that limits its growth and is not mutually exclusive. First, bacteria get iron by breaking down hemoglobin, such as hemolytic bacteria. FeII does not have enough time to oxidize to insoluble Fe III in this situation. Second, using a particular binding protein, the pathogen can bind to transferrin or lactoferrin. At the bacterial cell surface, the iron is then taken from the molecule. Third, the bacteria create a chelating chemical termed siderophore, which has a stronger affinity for iron than the host organism’s iron-containing molecules [22].

3. Microbial siderophores and *P. aeruginosa* siderophores

Bacteria possess specific pathogenicity mechanisms that they exhibit to overcome a host’s defenses. A pathogenic microorganism could cause damage, at any level, in a susceptible host organism. Virulence is a quantitative measure of pathogenicity and is measured by the number of microorganisms required to cause disease, that is, it is the degree of pathogenicity.

Throughout evolution, bacteria have acquired characteristics that allow them to invade the host environment, express specialized surface receptors for adhesion, remain in these sites through colonization processes, evade the immune system, and finally cause tissue damage within order to gain access to sources of nutrients necessary for their growth and reproduction [24, 25].

Therefore, the virulence factor or determinant is a microbial component that favors growth or survival during infection; iron being a determining factor of intracellular survival for the growth of most bacteria and especially pathogens, such as *P. aeruginosa*, an opportunistic human pathogen [26, 27].

1. When a microorganism enters a host organism, either in a pathogenic or symbiotic form, it finds a favorable environment with access to practically all the nutrients necessary for its growth except for one, iron. Iron, unlike other elemental sources

for nutrition, such as nitrogen, phosphorus, potassium, and other macro- and micronutrients, is not freely available in host organisms, so it is an important limiting factor for the growth of microorganisms. It is known that one of the responses of host organisms to pathogen attack consists in the reduction of free iron by sequestering this metal in ferritin molecules, structurally known as siderophores. This iron uptake mechanism that operates in bacteria has also been found in animals and plants. In the latter, there is a notable difference, and in the former, the control of ferritin synthesis occurs molecularly at the translational level, while in plants it occurs at the transcriptional level [4, 28–30], Hydroxamates: Siderophores that use a hydroxamate group to bind iron. The most representative siderophore is aerobactin, produced by bacteria of the Salmonella genus and some strains of E. coli, which has a dissociation constant very similar to transferrins, so it competes with other sources such as ferritin.

2. Cathecolates: Enterobactin is the most studied siderophore of this group, produced by strains of E. coli and other enterobacteria.

3. α-Hydroxycarboxylic acids: they are siderophores with a group similar to that of a hydroxamate, in which one of the radicals is replaced by a double bond with oxygen and nitrogen of the skeleton by a carbon. An example is the siderophore achromobactin produced by Erwinia chrysanthemi.

4. Mixed: those are in which two different binding groups are combined in the same molecule.

An example is anguibactin which contains a catechol and a hydroxamate group.

The siderophores, despite the variety in their structures, have similarities between them:

- They contain strongly electron donating atoms (often oxygen and, to a lesser degree, nitrogen or sulfur).

- Their shape is thermodynamically stable.

- They contain high Fe3+ spin species.

- They have a redox potential between −0.33 V (triacetylfusarinine) and − 0.75 V (enterobactin).

More than 500 siderophores, chemically characterized and classified, are currently reported. In addition, some have been shown to have the ability to chelate (subtract) other metals other than iron, such as aluminum, gallium, chromium, copper, zinc, lead, manganese, cadmium, vanadium, indium, plutonium, and uranium. Due to the great variety of siderophores, it is evident that several mechanisms of iron (III) transport exist [31, 32].

3.1 *P. aeruginosa* siderophores

P. aeruginosa synthesizes two types of siderophores, pyoverdine (PVD), and piochelin (PCH). Pyoverdine is the major siderophore of fluorescent pseudomonads

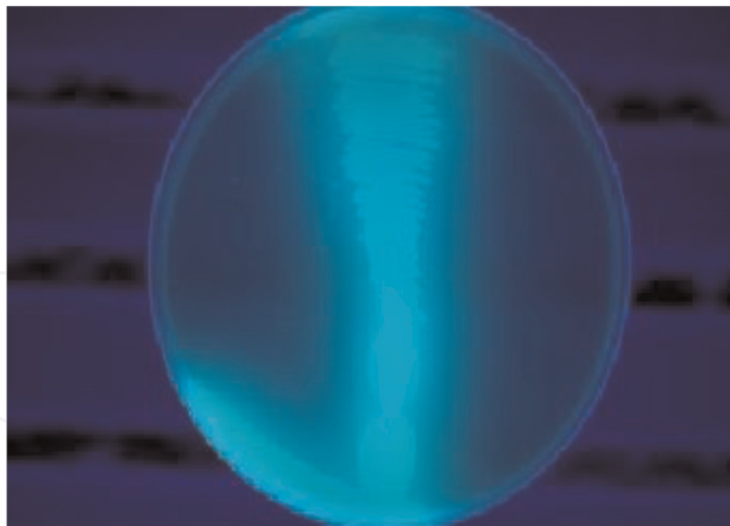

Figure 3.
P. aeruginosa *fluorescent pyoverdine growthing in an iron depleted media.*

(**Figure 3**). Pyoverdines were discovered in 1892, and over the years, they have been given various names: fluorescins, pseudobactins, and finally pyoverdins or pyoverdines. In 1952, J. Totter and F. Moseley observed that the iron levels affected the production of fluorescin by *Pseudomonas aeruginosa*. Today, more than 100 pyoverdines from different strains and species of *Pseudomonas* have been chemically identified and characterized [33]. Pyoverdine are mixed hydroxamate-catecholate siderophores, which are synthesized under iron-limited conditions. Pyoverdine has a high binding affinity for ferric iron. *P. aeruginosa* strains produce four distinct pyoverdines, called PVDI, PVDII, PVDIII, and PVDIV, and PCH as a secondary siderophore [34]. Each is characterized by a different peptide chain and each has a corresponding outer membrane receptor FpvAI, FpvAII, FpvAIII, and FpvAIV [34, 35]. Each outer membrane transporter can recognize and capture back only the ferric form of the produced pyoverdine or one that is structurally related (with a similar peptide sequence) by recognizing the peptide moiety. The methods to identify pyoverdines in different P. aeruginosa strain is referred to as siderotyping [36–40].

P. aeruginosa produces a second siderophore called pyochelin which has a lower binding affinity for the ferric form of iron (**Figure 4**). Pyochelin is the condensation product between salicydic acid and two cysteinyl residues. Pyochelin also chelates Zn (II), Cu(II), Co(II), Mn(II), and Ni(II) [38].

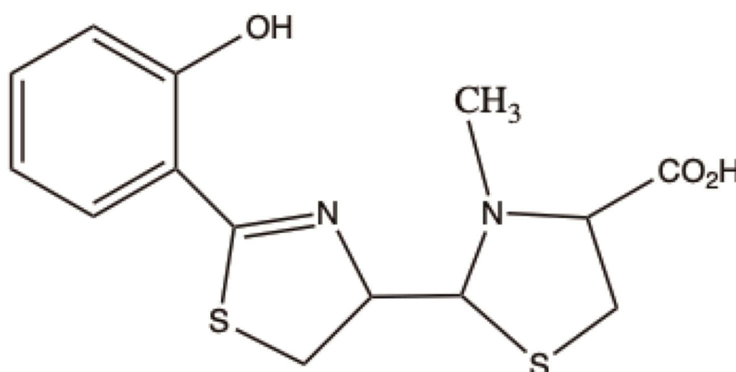

Figure 4.
Pyochelin of P. aeruginosa *PAO1 [41].*

4. Pyoverdine structure

Pyoverdines are a class of fluorescent yellow-green siderophores produced and secreted by many Pseudomonas species. In addition to pyoverdine, other siderophores with lower affinity for ferric ions are also produced such as pyochelin, pseudomonin, corrugatins, yersiniabactin, and thioquinolobactin [42]. Siderophores are small molecules not only produced by many microorganisms but also by plants whose molecular mass range from 200 to 2000 Da. These molecules are used to chelate iron with high affinity and functions in iron acquisition and also as virulence factors in some bacterial. The term siderophores from greek roots "sideros phoros" means iron carrier or transporter. There are different types of siderophores classified according to the ligand used to chelate iron. Catecholates are the more common functional group used to chelate iron in bacterial siderophores (i.e. enterobactin). Hydroxymates (i.e. Ferrioxamine B) are present in bacteria and Ferrichrome in fungi. Carboxylates (i.e. Rhizobactin) are present as functional groups in some bacterial siderophores; however, siderophores such as pyoverdine have a mix of functional groups that form hexadentate coordinates complexes with ferric iron [42]. Plants siderophores are called phytosiderophores, and the mugineic acid is the more common siderophore in plants. Pyoverdine siderophores molecules consist of a hydroxyquinoline chromophore core, a small peptide chain usually contain 6–14 amino acids and acyl side chain (**Figure 5**).

The chromophore is responsible for the color of the molecule and is linked to the peptide chain and acyl group. Both hydroxyl group of the chromophore and side chains oxygens in the peptide chain form interactions with iron. The peptide chain may be partially or completely cyclized and has L and D configuration amino acids. Unusual amino acids such as *N*5-formyl-*N*5-hydroxy ornithine, cyclo-*N*5-hydroxy ornithine, allo-threonine, and others may be present in the peptide chain. The amino acids compositions vary among Pseudomonas species and strains such as *Pseudomonas aeruginosa* strains such as ATCC27853, PAO1, and Pa6 (**Figure 6**). More than 100 different pyoverdines have been identified in Pseudomonas species and strains. Each pyoverdine has a peptide chain with a specific amino acid sequence and length [34, 39].

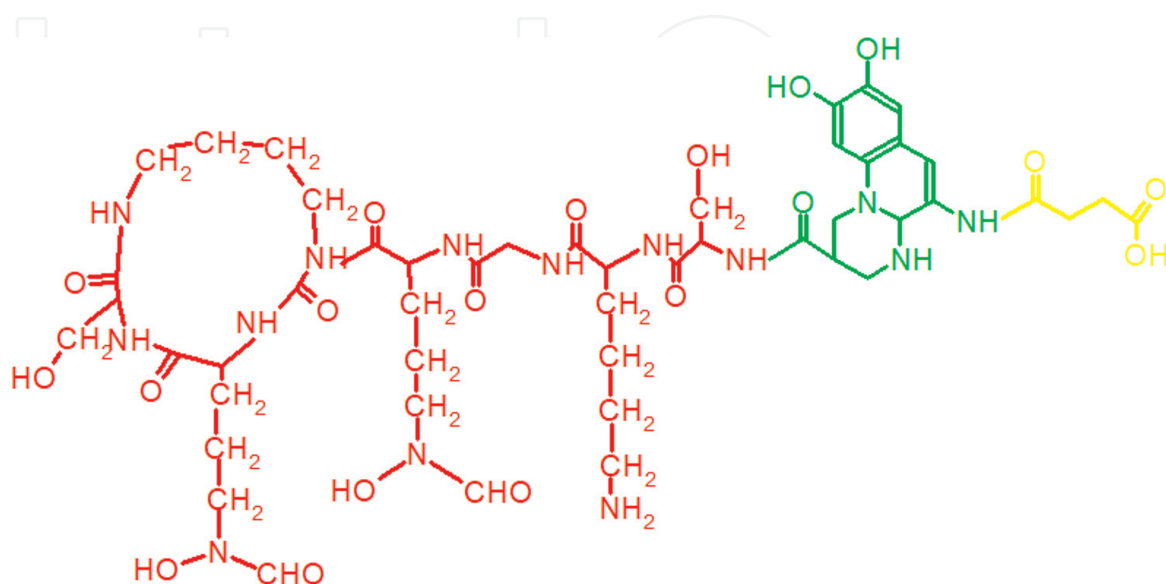

Figure 5.
Pyoverdine structure from P. aeruginosa *PAO1. Red, peptide chain partially cyclized. Green, dihydroxiquinoline chromophore showing the iron hydroxyl interacting groups. Yellow, acyl group [41].*

***P. aeruginosa* strains**	**Peptide chain components**	**PVD**
P. aeruginosa ATCC27853	**Ser**-fOH**Orn**-Orn-Gly-a**Thr**-Ser-(OHOrn)	**II**
P. aeruginosa PAO1	Glu-Tyr-**Dab**-Ser-Arg-Ser-fOHOrn-Lys-fOHOrn-Thr-Thr	**I**
P. aeruginosa Pa6	**Ser**-Dab- fOHOrn-Gln-**Gln**-fOH**Orn**-Gly	**III**
P. aeruginosa R'	**Ser-**Dab-fOHOrn-Gln-fOHOrn-Gly	**IV**

Figure 6.
Peptide chain composition of three pseudomonas strains. Amino acids in bold are D configurations. Cyclic structure in the chain is in parenthesis. fOHOrn is N^5-formyl-N^5-hydroxyornithine. aThr correspond to Allo-threonine and dab is L-2,4- diaminobutyrate. The acyl side chain (amide or dicarboxylic acid) is linked to the amino group of the chromophore. The length and type of acyl side chain depend on strain and growth conditions and whose purpose remains unclear [34, 39, 43, 44].

5. Pyoverdines biosynthesis and transport

The siderophores biosynthesis is a complex enzymatic process that requires several specific enzymes whose expression is regulated by iron and different transcriptional factors. The enzymes involved in siderophores biosynthesis are organized into a multi-enzymatic complex, called siderosomes, and are in close vicinity to each other in the cytoplasmic face of the inner membrane. This organization may reduce the diffusion of siderophores precursor. Most of the siderosome enzymes have modular and each module incorporated specific amino acids into a growing peptide chain. Enzymes involved in the biosynthesis of unusual amino acids present in siderophores are also proposed to be part of the siderosome (**Figure 7**) [45].

Pseudomonas aeruginosa strains have four types of pyoverdines designated as PVD1, PVDII, PVDIII, and PVDIV and the biosynthesis of pyoverdine I (PVDI) in *P. aeruginosa* PAO1 involved the action of seven siderosome enzymes. The same number of enzymes is required for pyochelin biosynthesis. A siderosome model for P. fluorescens strain A506 has been included (**Figure 7**).

The initial step in pyoverdines biosynthesis takes place in the cytoplasm where non-ribosomal peptide synthetases (NRPSs) catalyze the formation of the peptide

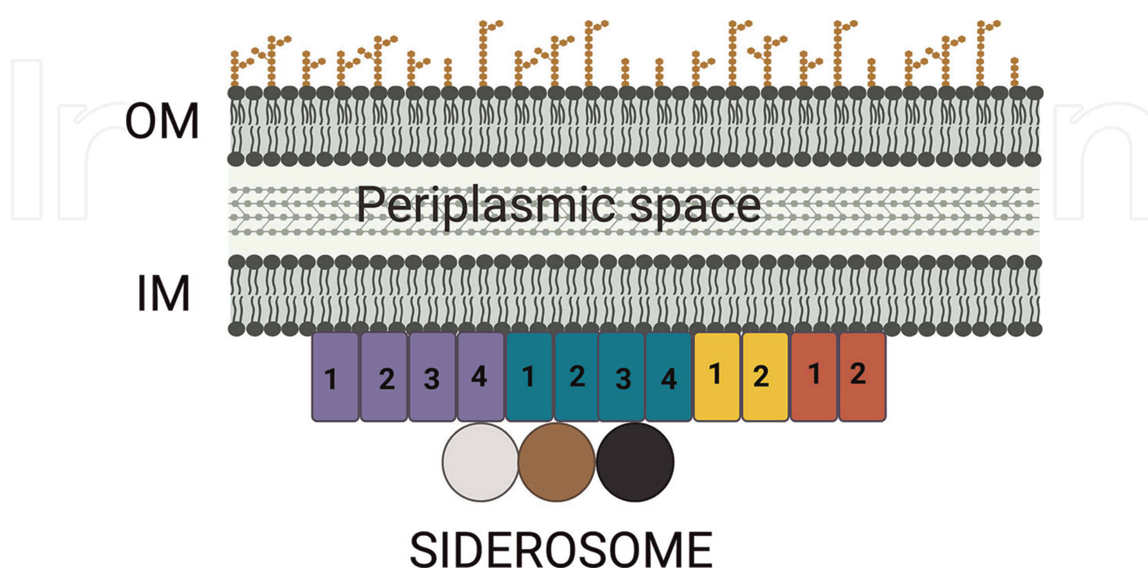

Figure 7.
Model of siderosome of P. aeruginosa *PAOI. The modular enzymes such as PvdL, PvdI, PvdJ, and PvdD are shown in blue, green, yellow, and red color, respectively. The modules in each enzyme are numbered. PvdH, (gray) PvdA (brown), and PvdF (black) are non-modular siderosome enzymes involved in pyoverdine synthesis. OM, outer membrane and IM, inner membrane [45]. Created with BioRender.com.*

precursor for pyoverdines called acylated precursor chain (**Figure 8**) [46, 47]. *P. aeruginosa* PAO1 has four non-ribosomal peptide synthesis (NRPS) enzymes: PvdL, PvdI, PvdJ, and PvdD [48]. Three more enzymes are involved in the formation of unusual amino acids of the peptide chain of PVDI. The unusual amino acids in PVDI are L-2,4 diaminobutyrate (Dab) and L-N5-formyl-N5-hydroxyornithine (fOHOrn). The PvdH enzyme is responsible for the synthesis of Dab, PvDA, and PvDF that catalyze the synthesis of fOHorn through consecutive hydroxylation and formylation reactions. Model of siderosome of *P. fluorescens* strain A506 includes three NRPS enzymes (PvdG, PvdL, and PvdD) and the same *P. aeruginosa* enzymes that catalyze the formation of unusual amino acids.

The NRPS enzymes are modular enzymes with 2–4 modules. PvdL and PVdI have four modules and PvdJ and PvdD are bimodular. The first module (M1) of PvdL catalyzes the incorporation of acyl group (myristic or myristoleic acid) instead of amino acid. This acylation probably links the peptide to the membrane and prevents diffusion during synthesis. The M2 of PvdL catalyzes the activation of L-Glu and its condensation to the acyl group. PvdL, module three (M3), incorporates an L-Tyr that is converted to D-Tyr by domain of this module. M4 adds Dab to generate an acylated tripeptide (Glu-Tyr-Dab). PvdI modules are responsible for adding D-Ser, L-Arg, D-Ser, and fOHOrn to previous acylated tripeptide. L-Lys and fOHorn and two L-Thr are, respectively, added by the bimodular enzymes PvdJ and PvdD. The peptide bound formation is catalyzed by a PCP domain present in the modules. Thioesterase domain of the PvdD module is released by hydrolysis of the 11 amino acid chain from the NRPS [42].

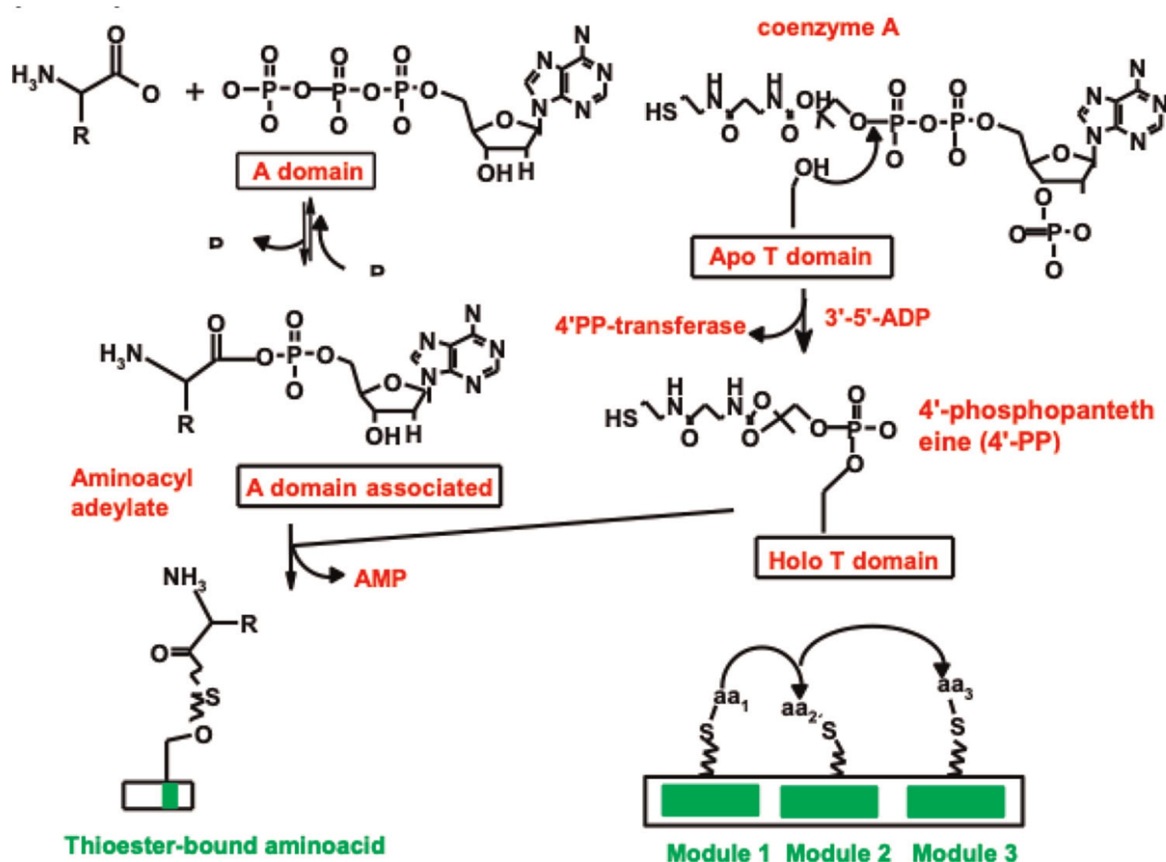

Figure 8.
Mechanism of multiple carrier thiotemplate [47].

The released peptide is transported to the periplasmic space where it is modified. The transport to the periplasmic space involved a class of ABC pumps codified by *pvdE* gene. The modifications that take place in the periplasmic space includes the deacetylation and the removal of myristic or myristoleic acid from the peptide chain and the formation of pyoverdine precursor called ferribactin. The PvdQ enzyme is responsible for this modification. The enzyme PvdP converts ferribactin into dihydropyoverdine. The conversion of dihydropyoverdine to PVDI is catalyzed by the enzyme PvdO.

Finally, the PVDI is secreted from the periplasmic space to the environment via PvdRF-OpmQ ATP pump. The secreted PVDI binds to ferric iron to form PVDI-iron complex (Ferripyoverdine). The Ferripyoverdine is imported via FpvA receptors which interact with TonB-ExbBD complex and the help of transporter FpvB [49]. The Fe^{+3} of the Ferripyoverdine in the periplasmic space is reduced to Fe^{+2} and released from pyoverdine. Liberated Fe^{+2} is transported into the cytoplasm through ABC transporter FpvDE.

6. Regulation of pyoverdine production

The transcriptional control of genes involved in the synthesis of pyoverdine is induced by iron deficiency or depletion (**Figure 9**). The regulation of pyoverdine production involves sensing cytoplasmic levels of iron ions by the regulator protein Fur, which in turn represses regulatory genes involved in iron uptakes, such as FpvR, FpvI, and PvdS [50–54]. PvdS is a sigma factor required for the expression of pyoverdine biosynthesis genes and some virulence-related genes [55–58, 29]. FpvI is a sigma factor required by the genes encoding the outer membrane pyoverdine receptor/importer FpvA, and FpvR is an anti-sigma factor that binds to and inactivates PvdS and FpvI [50, 59]. FpvR autoproteolytic cleaves itself at a periplasmic domain without any further degradation unless it contacts ferripyoverdine-bound FpvA. When FpvR/FpvA contact occurs, which involves the activity of TonB (the transport-energizing inner membrane protein), the protease RseP releases PvdS and FpvI allowing the activation of their regulated genes [50, 60]. The regulation of pyoverdine biosynthesis is more complex because it involves signals other than iron starvation, such as the influence of the regulator protein CysB may imply coordination with sulfur availability or biofilm formation and alginate production [61, 62]. Phosphate starvation has been reported to trigger pyoverdine production in host environments [63]. Additionally, the LexR-type transcriptional regulator AmpR affects the expression of more than 500 genes related to metabolism and virulence in *P. aeruginosa* and has been recently implicated in the regulation of pyoverdine production [64]. Intracellular levels of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) may also modulate pyoverdine production [65].

7. Pyoverdine as virulence factor in *Pseudomonas aeruginosa*

The World Health Organization classified *P. aeruginosa* as a priority one pathogen due to the multidrug resistance mechanisms of this bacterium. As we mentioned, P. aeruginosa produces diverse and overwhelming infections due to the wide variety of

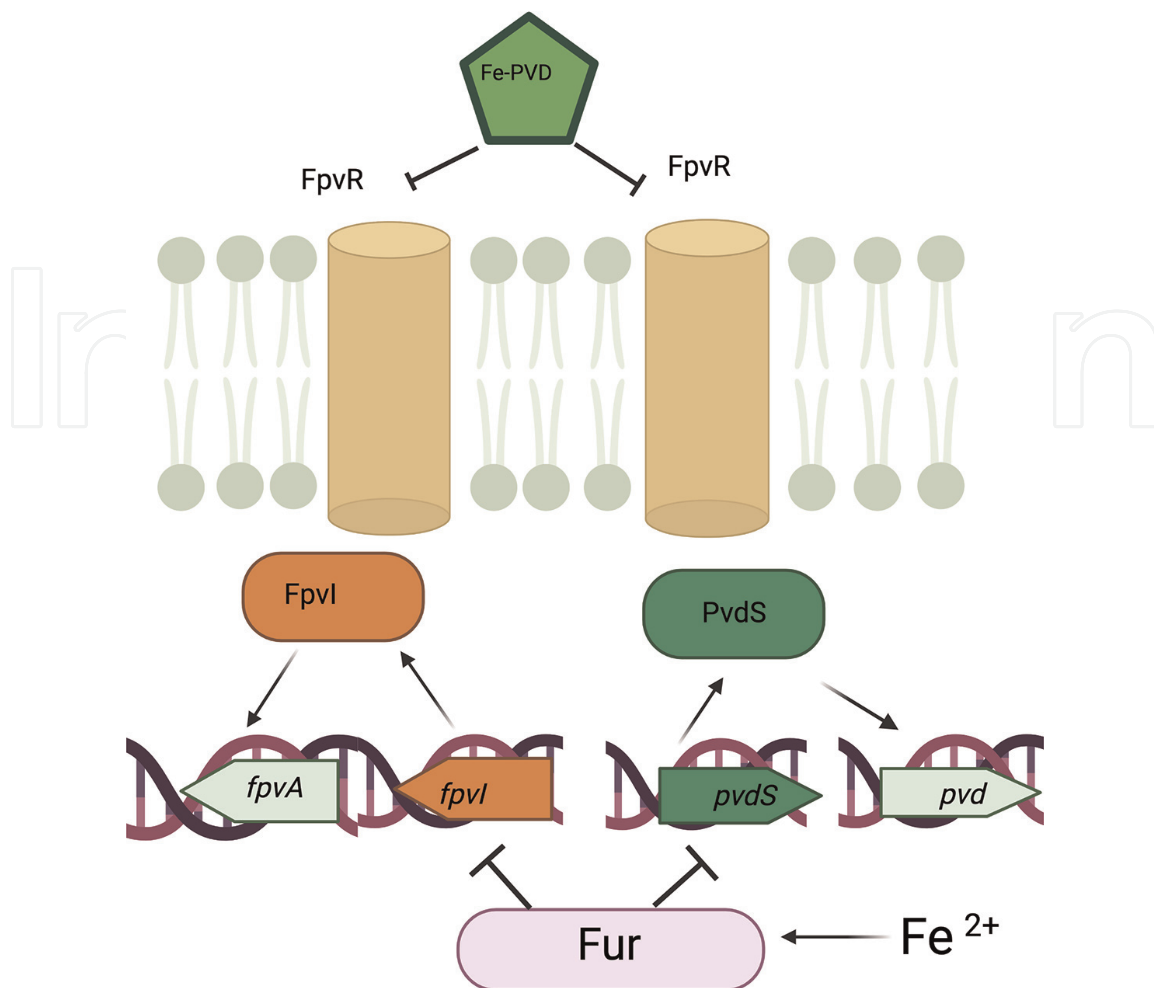

Figure 9.
The ferripyoverdine signaling pathway. The alternative sigma factors σ^{FpvI} and σ^{PvdS} direct expression of the fpvA *gene encoding the cell surface ferripyoverdine receptor protein FpvA, and* pvd *genes encoding enzymes required for synthesis of pyoverdine. σ^{FpvI} and σ^{PvdS} are inhibited by the $FpvR_{20}$ protein, which is degraded in the presence of ferripyoverdine (Fe-Pvd). Expression of the* fpvI *and* pvdS *genes is repressed by the iron (Fe^{2+})-containing form of the fur repressor [66]. Created with BioRender.com.*

virulence factors that are responsible for multidrug resistance strains (MDRs) [67]. The pyoverdine siderophore is a key virulence factor that provides the bacterium with iron during infection [68].

Regarding the virulence, it has been found that deficient pyoverdine mutants of *P. aeruginosa* were not virulent in the Burn mouse model compared with the wild-type strain PAO1. The mutant strain virulence was restored when pyoverdine purified from the wild-type strain was added during infection. In a similar study, although infected calf muscles of immunocompromised mice were injected with pyoverdine and pyochelin mutants, no lethality was observed, concluding the role of pyoverdine during infection. The pyoverdin-deficient mutant and the double mutant, on the other hand, grew poorly in the lungs when compared to wild-type strain PAO1, and the latter's virulence was significantly reduced [69, 70].

In the model nematode Caenorhabditis elegans, pyoverdine is virulent, even in the absence of the pathogen. A study found that when this siderophore is consumed by C. elegans together with other chemicals in its aqueous environment, pyoverdine gains access to and eliminates ferric iron through an unknown method once within the host. The host mitochondria, which are iron-rich organelles, are a likely target for this

abstraction. Mitochondrial function is disrupted, and mitochondria are targeted for turnover when they are removed. In vitro experiments with pyoverdine-treated murine macrophages revealed considerable toxicity, while no pyoverdine production reduced pathogenicity. Furthermore, pyoverdine translocates into cells and impairs host mitochondrial homeostasis, as previously observed in C. elegans [71–73].

Pyoverdine is a multifaceted role in *P. aeruginosa* pathogenesis; in addition, scavenging iron from host proteins also regulates the expression of several virulence factors, including exotoxin A, an endoprotease PrpL, and pyoverdine biosynthesis itself acting as a signaling molecule to control the production of secreted products [74]. A recent study using a double mutation in genes involved in biofilm formation showed that pyoverdine is essential for the development of virulent factors such as exotoxin A and PrpL protease [4, 75].

Exotoxin A is one of *P. aeruginosa's* most potent toxins, capable of inducing apoptosis in host cells and killing model organisms [76]. Under iron-repleted growth conditions, the transcription of exotoxin A was absent, indicating that it is negatively regulated for iron. Interestingly, in the presence of iron ions, pyoverdine was found to activate a signaling pathway for the upregulation of exotoxin A expression [77, 78].

The extracellular protease IV, PrpL, degrades surfactant proteins and interleukin-22 necessary for pulmonary mucosal immunity that made *P. aeruginosa* a major pathogen of ventilator-associated pneumonia and causes considerable lung tissue damage [79].

The sigma factor PvdS is required for the expression of PrpL. The extracellular protein profiles obtained, using PAO1 and a Δ*pvdS*::*Gm* mutant, showed that PrpL ((PvdS-regulated endoprotease, lysyl class) was expressed under the control of PvdS under iron-deficient conditions. In this study, Rnase protection assays confirm that the initiation of transcription is iron-dependent. A study shows that expression of prpL was lower in the biosynthetic gene pvdF mutant than in wild-type bacteria, and expression was increased to wild-type levels by the addition of pyoverdine [80].

The relationship between iron and antibiotic resistance in *P. aeruginosa* has been reviewed [21]. It is required to determine whether iron fluctuations are a critical component for antibiotic resistance in this bacterium, according to this review. A series of studies concluded that increased concentration of iron in the growth medium decreased the resistance of *P. aeruginosa* strains of various origins to antibiotics such as ampicillin, norfloxacin, gentamicin, ofloxacin, and cefsulodin [21]. Conversely, another study showed that increasing iron concentration increased resistance to two antibiotics, tobramycin and tigecycline by using the wild-type strain PAO1 and isolated strains from cystic fibrosis patients in a growth medium with an iron chelator, chelex, and $FeCl_3$ (100 μM) [81]. Inversely, Singh and collaborators found that "decreasing" iron concentration "decreased" resistance to tobramycin [82]. They used an iron chelator, the most used being 2,2 dipyridyl (DIP) or deferoxamine (DFO), a siderophore used by P. aeruginosa to the growth medium, to create conditions of iron limitation. These discrepancies could be attributable to the variability of experimental methods, particularly the iron concentration settings and in addition P. aeruginosa uptake iron by different methods and those are important for antibiotic resistance mechanisms.

Therefore, pyoverdine plays an important role in antibiotic resistance, since it mediates the uptake of iron in *P. aeruginosa*. Oglesby-Sherouse et al. [81] demonstrated that pyoverdine increases the ability of *P. aeruginosa*to resist tigecycline treatment. A recent study with clinical isolates of P. aeruginosa showed the importance of pyoverdine for the bacteria virulence, since most of them were MDR-expressing resistance such as genes of the MexAB-OprM efflux pump system (mexABR) and pyoverdine receptor genes (fpvA) which are induced by iron limitation conditions.

Among the clinical isolates, 22 out 51 were ESBLs (extended-spectrum β-lactamases) producers which represent an important subclass of enzymes that confer resistance to oxyimino-cephalosporins [83]. A similar study related the pyoverdine production, biofilm formation, ESBL, and other virulence genes (OprI, OprL, LasB, PlcH, ExoS, and ToxA) with the antibiotic resistance of 54 clinical isolates, and among them 93% were pyoverdine producers showing its role in antibiotic resistance [83]. A strong relationship between EBSL isolates producers and virulence factor, including pyoverdine, was also found in clinical isolates of P. aeruginosa [84]. Additionally, multidrug resistance, presence of virulence-associated genes, and expression of certain virulence factors, most notably elastase, protease, siderophore, and DNase activity, were strongly related to pyoverdine production. A recent study with clinical isolates of P. aeruginosa related its pathogenicity with pyoverdine production in model's organism in *C. elegans* and an acute murine pneumonia model [85].

8. Concluding remarks

The rise of resistant *P. aeruginosa* strains in hospitals has prompted researchers to look for new therapeutic options. Attenuation of the pathogen's virulence factors is one of these strategies. The pathophysiology of the bacteria is restricted because it does not need to be eliminated or killed. In many cases, attenuation of these products that harm the host is unnecessary for growth or colonization, and their absence causes the pathogen to adopt a commensal lifestyle. Pyoverdine is an important virulence factor, and its role in iron transport as well as its position as a signaling molecule for the synthesis of other virulence factors make it an attractive target for new therapeutics that block its function. A potential therapeutic method involves tagging pyochelin or pyoverdine with an antibiotic or a redox-inactive metal ion such as gallium, which interferes with P. aeruginosa iron metabolism and its synthesis. Some *P. aeruginosa* strains may utilize another iron-chelating pathway using nicotianamine in the absence of siderophores, which may be investigated in the same way.

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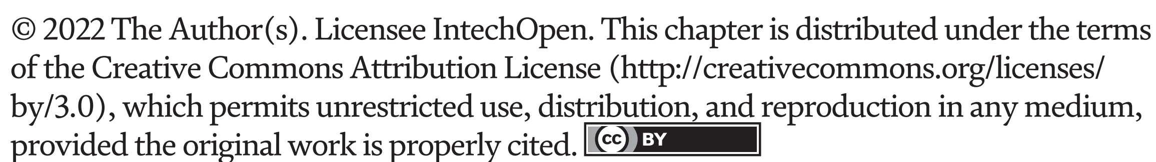

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