Navigating pyoverdine and beyond: The role of tripartite efflux pumps in *Pseudomonas putida* KT2440

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Nicola Victoria Maria Stein

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Gutachter:

1. Professor Dr. Heinrich Jung

2. Professor Dr. Simon Heilbronner

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Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde. Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die folgende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

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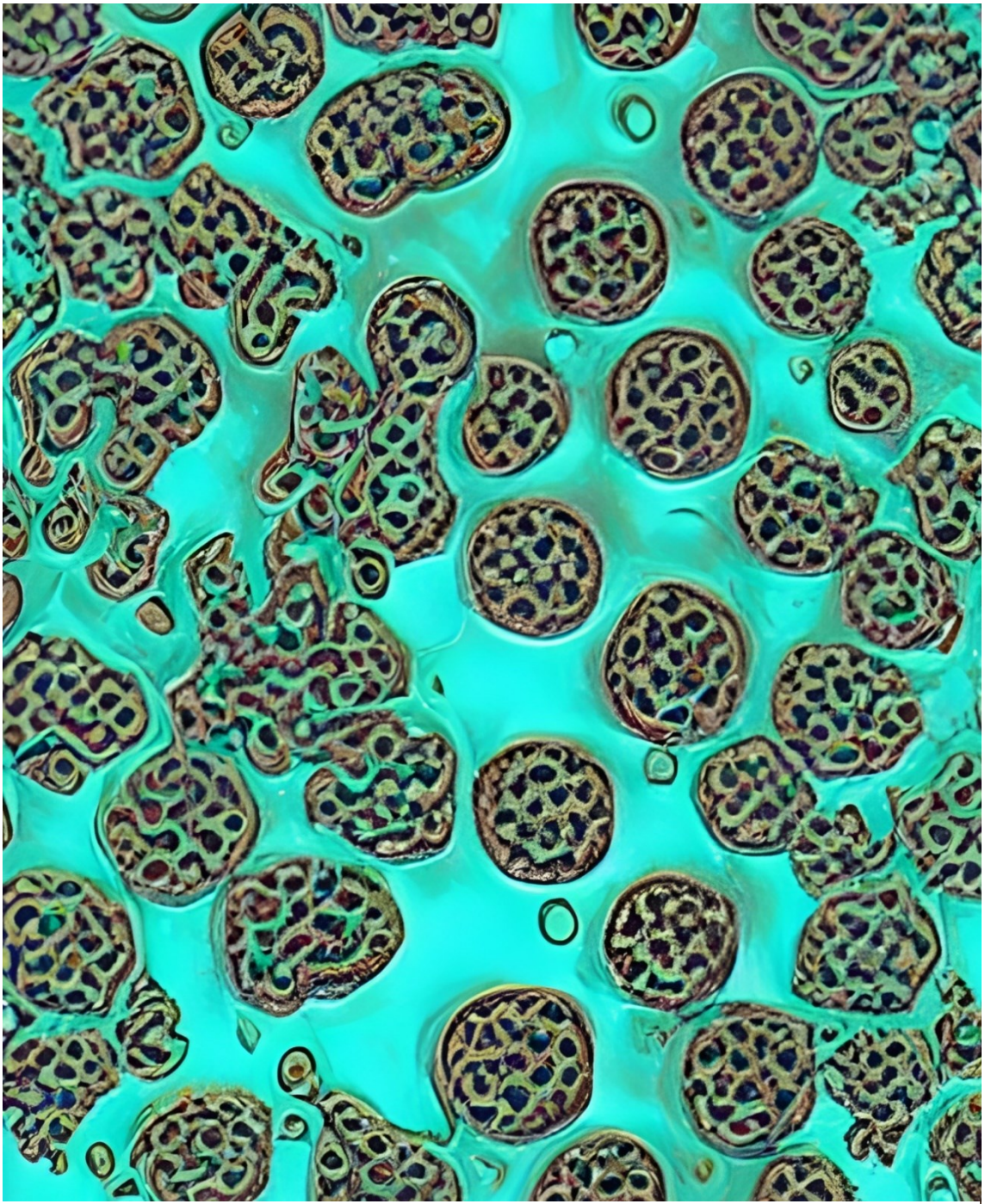
Für Carolin

“Never be limited by other people’s limited imaginations.”

Dr. Mae Jemison



- Bacterium, cyan, fluorescent, iron –

The period of this work was marked by the release of publicly available artificial intelligence software to everyone. This raises questions about the benefits and risks of such developments for humanity, especially for research. Nevertheless, curiosity was greater than fear. This illustration is the result of an artistic AI inspired by the theme of this work, using the keywords: bacterium, cyan, fluorescent, and iron. The original output is shown as it was created with deepAI.org.

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Nomenclature

Genes are given with the respective identifier, starting with PP and the gene number in the genome for *Pseudomonas putida* KT2440 (NCBI BioSample Accession number SAMN02603999). The abbreviation PA was used for genes of *Pseudomonas aeruginosa* PAO1 (NCBI BioSample Accession number SAMN02603714). Respective deletions are labeled with Δ. If available, annotated gene names are given (e.g., PvdT). To simplify the reading process in this thesis, especially the discussion (Chapter 5), proteins were labeled with the organism identifier if applicable and abbreviated as such after first mention in the body text (e.g., *Ec*MacAB stands for MacAB from *Escherichia coli*, *Pp*PvdRT stand for PvdRT from *Pseudomonas putida* and *Pa*MexAB stand for MexAB from *Pseudomonas aeruginosa*).

Abbreviation

ABC – ATP binding cassette

Bip – 2,2’ bipyridyl (ion chelator)

Bp - Basepair

Ec – *Escherichia coli*

IEF - Isoelectric focusing

IM – Inner membrane

IMP – Inner membrane protein

MATE – Multidrug and toxin extrusion

MDR – Multidrug-resistant

MFP – Membrane fusion protein

MFS - Major Facilitator Superfamily

OM – Outer membrane

OMP – Outer membrane protein

PA/*Pa* – *Pseudomonas aeruginosa*

PACE - Proteobacterial Antimicrobial Compound Efflux

PAH - Polycyclic aromatic hydrocarbon

PHA - Polyhydroxyalkanoates

Pmf – Proton motive force

PP/*Pp* – *Pseudomonas putida*

PVD - Pyoverdine

ROS – Reactive oxygen species

SMR - Small Multidrug Resistance

Smf- Sodium motive force

Publications and manuscripts originating from this thesis

Chapter 2: The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization

Nicola Victoria Stein, Michelle Eder, Sophie Brameyer, Serena Schwenkert and Heinrich Jung (2023). The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization *FEBS Lett.* 597, 1403-1414, DOI: 10.1002/1873-3468.14601

Chapter 3: Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440

Tania Henríquez, Nicola Victoria Stein and Heinrich Jung (2020). Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440. *Front. Microbiol.* 11, 1974, DOI: 10.3389/fmicb.2020.01974

Chapter 4: The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440

Nicola Victoria Stein, Michelle Eder, Fabienne Burr, Sarah Stoss, Lorenz Holzner, Hans-Henning Kunz and Heinrich Jung. The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440. *Microbiology Spectrum*, e02300-23, DOI*:* 10.1128/spectrum.02300-23

Further publications not originating from this dissertation and not included

Tania Henríquez, Nicola Victoria Stein and Heinrich Jung (2019). PvdRT-OpmQ and MdtABC-OpmB efflux systems are involved in pyoverdine secretion in *Pseudomonas putida* KT2440*. Environ. Microbiol. Rep.* 11 (2019), 98-106, DOI*:* 10.1111/1758-2229.12708.

Contributions to publications and manuscripts presented in this thesis

Chapter 2: The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization

All experiments were performed by Nicola Stein, Michelle Eder, and Sophie Brameyer. Nicola Stein and Michelle Eder generated overexpression systems and purified protein. Nicola Stein performed DRaCALA, SEC, and native gel protein analyses. Michelle Eder performed ATPase assays. Sophie Brameyer performed SPR measurements with purified protein and subsequent analysis. Serena Schwenkert performed Mass spectrometry and analysis. Nicola Stein and Heinrich Jung designed the study.

Chapter 3: Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440

Tania Henríquez and Nicola Stein performed all experiments. Tania Henríquez generated deletion mutants and performed growth analysis in iron-deplete and replete conditions. Nicola Stein generated respective complementation strains and performed growth analysis in iron-deplete conditions. Tania Henríquez analyzed pyoverdine concentrations and did colony morphology assays. Tania Henríquez measured intracellular ATP levels and performed gene expression analysis (qRT-PCR). Nicola Stein performed resistance testing towards deoxycholate and bile salts and performed fluorescence microscopy with image analysis. Tania Henríquez analyzed the influence of iron and copper supplementation. Tania Henríquez, Nicola Stein, and Heinrich Jung designed the study.

Chapter 4: The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440

Nicola Stein, Michelle Eder, Fabienne Burr, and Sarah Stoss generated the strains and plasmids. Nicola Stein, Michelle Eder, Fabienne Burr, and Sarah Stoss performed growth measurements in iron-deplete and replete conditions. Nicola Stein and Sarah Stoss performed pyoverdine measurements. Nicola Stein performed microscopy and image evaluation. Nicola Stein and Fabienne Burr performed the luciferase assays for promotor fusions. Nicola Stein, Michelle Eder, and Fabienne Burr performed susceptibility tests and rescue experiments with iron, copper, and pyoverdine. Lorenz Holzner and Hans-Henning Kunz performed TXRF measurements for iron determination. Nicola Stein and Heinrich Jung designed the study.

We, with this, confirm the above statements:

Nicola Stein	Prof. Dr. Heinrich Jung

Summary

Pseudomonads produce siderophores to survive in challenging environmental conditions. Siderophores are small molecules that chelate iron ions (Fe^{3+}) and enable the uptake into the cell. The processes involved – siderophore synthesis, maturation, uptake, and recycling - are well understood, while the mechanisms of siderophore secretion still need to be investigated. In the soil bacterium *Pseudomonas putida* KT2440, two systems have been identified that contribute to siderophore secretion: the tripartite efflux systems PvdRT-OpmQ and MdtABC-OpmB. It is believed that these systems enable the secretion of pyoverdine, the fluorescent siderophore of pseudomonads, into the environment by binding mature pyoverdine in the periplasm and transporting it through the outer membrane of the Gram-negative cell. PvdRT-OpmQ belongs to the ATP-dependent ATP-binding cassette (ABC) transporter family, whereas MdtABC-OpmB is a proton motive force-dependent member of the resistance-nodulation-cell division (RND) transporter family. Inactivation of both systems inhibits but does not entirely block the secretion of pyoverdine, suggesting that other systems also contribute to the secretion process. Moreover, evidence for the participation of the systems in pyoverdine secretion comes from gene deletions and phenotypic analyses, whereas biochemical evidence for the interaction of protein complexes with the cognate substrate is lacking.

The aim of the thesis was to clarify the molecular basis of the secretion of the siderophore pyoverdine in pseudomonads, using *P. putida* KT2440 as a model. On the one hand, the focus was on identifying possible additional transport systems involved in pyoverdine secretion. On the other hand, biochemical studies were performed to provide the first information on the interaction of pyoverdine with the proposed transport systems.

Following a comprehensive screening of transporters, the RND efflux system ParXY was shown to affect pyoverdine secretion. Deletion of the gene of the periplasmic adapter protein ParX inhibited growth under iron-limiting conditions and secretion of the siderophore when PvdRT-OpmQ and MdtABC-OpmB were inactivated. The effects observed depended on the presence of other tripartite efflux systems and the amount of available iron in the growth medium. The results indicate that ParXY contributes to antibiotic resistance and impacts siderophore secretion through a complex network involving various tripartite efflux systems. Furthermore, the PvdRT-OpmQ system was biochemically characterized. For this purpose, the inner membrane component PvdT and the periplasmic adapter protein PvdR were purified. PvdT was shown to possess an

ATPase activity that was stimulated by the addition of PvdR. The first biochemical evidence for direct interactions between pyoverdine and PvdRT was obtained. In addition, information on the role of the RND system TtgABC in the response to toxic substances was expanded.

The results of this work not only provide insights into siderophore secretion in pseudomonads and contribute to a better understanding of the importance and functioning of the tripartite efflux systems, which are also known for their role in antibiotic resistance.

Zusammenfassung

Pseudomonaden produzieren Siderophore, um unter schwierigen Umweltbedingungen überleben zu können. Siderophore sind kleine Moleküle, die Eisen-Ionen (Fe^{3+}) chelatisieren und so die Aufnahme in die Zelle ermöglichen. Die beteiligten Prozesse - Synthese, Reifung, Aufnahme und Recycling - sind gut untersucht, während die Mechanismen der Siderophorsekretion noch weiter erforscht werden müssen. In dem Bodenbakterium *Pseudomonas putida* KT2440 wurden bisher zwei Systeme identifiziert, die zur Siderophorsekretion beitragen: die dreiteiligen Effluxsysteme PvdRT-OpmQ und MdtABC-OpmB. Es wird angenommen, dass diese Systeme die Sekretion von Pyoverdin, dem fluoreszierenden Siderophor der Pseudomonaden, in die Umwelt ermöglichen, indem sie reifes Pyoverdin im Periplasma binden und durch die äußere Membran der gramnegativen Zelle transportieren. PvdRT-OpmQ gehört zur Familie der Transporter, die eine ATP-bindende Cassette besitzen (ABC-Transporter), während MdtABC-OpmB zu der Familie RND-Transporter (*Resistence-nodulation cell division*) gehört, welche von der protonenmotorischen Kraft abhängig sind. Die Inaktivierung beider Systeme hemmt die Sekretion von Pyoverdin, blockiert sie aber nicht vollständig, was darauf hindeutet, dass weitere Systeme zum Sekretionsprozess beitragen. Darüber hinaus stammen die Beweise für eine Beteiligung der Systeme an der Pyoverdinsekretion aus Gendeletionen und phänotypischen Analysen, während biochemische Nachweise für die Interaktion des Proteinkomplexes mit dem assoziierten Substrat fehlen.

Das Ziel dieser Arbeit war es, die molekularen Grundlagen der Sekretion des Siderophors Pyoverdin in Pseudomonaden am Beispiel von *P. putida* KT2440 zu klären. Der Fokus lag zum einen auf der Identifizierung möglicher zusätzlicher Transportsysteme, die an der Pyoverdinsekretion beteiligt sind. Zum anderen sollten biochemische Untersuchungen erste Informationen über die Interaktion von Pyoverdin mit den bereits bekannten Transportsystemen liefern.

Nach einem umfassenden Screening von Transportermutanten wurde gezeigt, dass das RND-System ParXY die Sekretion von Pyoverdin beeinflusst. Die Deletion des Gens für das periplasmatische Adapterprotein ParX hemmte das Wachstum unter eisenlimitierenden Bedingungen und zusätzlich die Sekretion des Siderophors. Die beobachteten Effekte waren abhängig von der Anwesenheit anderer dreiteiliger Transportsysteme und den Bedingungen der Eisenlimitierung. Die Ergebnisse deuten darauf hin, dass ParXY nicht nur eine Rolle bei der Antibiotikaresistenz spielt, sondern

auch die Sekretion von Siderophoren in einem Netzwerk sich überschneidender Aktivitäten verschiedener dreiteiliger Effluxsysteme beeinflusst. Außerdem wurde das PvdRT-OpmQ-System biochemisch charakterisiert. Zu diesem Zweck wurden die innere Membrankomponente PvdT und das periplasmatische Adapterprotein PvdR gereinigt. Es wurde gezeigt, dass PvdT eine ATPase-Aktivität besitzt, die durch die Zugabe von PvdR stimuliert wurde. Es wurde der erste biochemische Nachweis für direkte Wechselwirkungen zwischen Pyoverdin und PvdRT erbracht. Darüber hinaus wurden die Informationen über das Substratspektrum des RND-Systems TtgABC in Bezug auf toxische Substanzen erweitert.

Die Ergebnisse dieser Arbeit geben nicht nur Einblicke in die Siderophorsekretion bei Pseudomonaden, sondern tragen auch zu einem besseren Verständnis der Bedeutung und Funktionsweise ebendieser dreiteiligen Effluxsysteme bei, die auch eine signifikante Rolle bei Antibiotikaresistenzen spielen.

1. Introduction

1.1 The group of pseudomonads and *Pseudomonas putida* KT2440 as a model organism

Pseudomonads are a diverse group of Gram-negative, straight or rod-shaped bacteria found in various environments [1-3]. According to the LPSN (*List of Prokaryotic Names with Standing in Nomenclature*), there are 478 validly published species among the very diverse genus of pseudomonads (accessed 19.12.2022) [4]. These bacteria are known for their ability to grow under challenging conditions [5]. Many pseudomonads are fluorescent [5] (Figure 1, top), and they develop between one and seven flagella (Figure 1, bottom). In general, the majority of pseudomonads possess only a single polar flagellum [3, 6]. The size of a single *Pseudomonas* cell generally ranges from 0.5 to 0.8 µm and 1.5 to 5 µm, whereas they can form huge communities in the form of biofilms [3, 7]. They are mostly oxidase and catalase positive [8]. Some strains of *Pseudomonas* are opportunistic [9] pathogens that exploit immunocompromised hosts, while others are obligate pathogenic to their hosts [8, 10, 11]. They occur in nosocomial infections and have been isolated from cancer, cystic fibrosis, or superficial burns patients. In addition, pseudomonads can cause infections of the central nervous system, urinary tract, eyes, and ears. More than 25 species of *Pseudomonas* have been isolated from humans [7, 12]. Other strains of *Pseudomonas* are pathogenic to plants, such as *P. syringae*, a common causative agent of bacterial canker in fruit trees, to insects, such as *P. entomophila* [2], or to fish, as recently demonstrated for the pathogenic *P. tructae* [10].

In contrast to these pathogenic isolates, some pseudomonads, such as *P. fluorescens*, can protect plants by preventing root rot and leaf spot in crops such as beans, corn, and tomatoes and protecting them from parasitic fungi colonization [13]. Pseudomonads can harbor different classes of plasmids that confer resistance towards antibiotics, chemicals, bacteriocins, and bacteriophage propagation. Other plasmids encode degradative pathways, for instance, for pollutants such as octanol (OCT plasmid), xylene (XYL,) or toluene (TOL). Besides, *Pseudomonas* are well known for their ability to degrade several unrelated compounds, such as hydrocarbons [3, 8].

The organism of interest in this thesis is *Pseudomonas putida* strain KT2440, which was recently classified in the *Pseudomonas alloputida* group (DSM 6125) [14, 15]. However, the strain will be referred to as *P. putida* KT2440 for this thesis. The upper panel of Figure

1 shows a micrograph of the wild-type strain (WT). It is an environmentally saprophytic soil bacterium and one of the most commonly used microorganisms for bioproduction and bioremediation [16, 17]. The strain is considered a valuable model organism in research as it is certified as a biosafety strain [18]. In nature, this strain is found in soils and plant rhizospheres and is part of freshwater communities [16, 19]. It is obligate aerobic, with the ability to cope with temporary oxygen limitation [17]. Oxygen functions as the primary terminal electron acceptor for respiration, with some exceptional strains also utilizing nitrogen as an electron acceptor [3, 8].

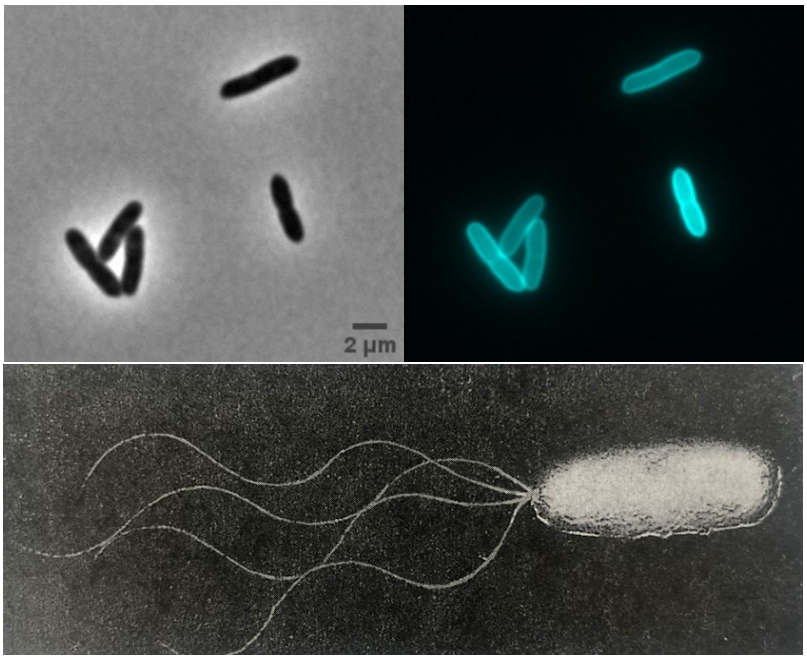


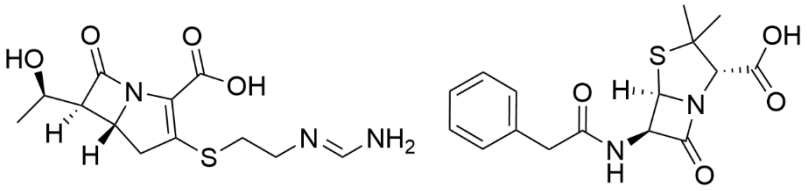
Figure 1: **Microscopic images of *Pseudomonas putida* KT2440 wildtype strain (top) and *Pseudomonas aeruginosa* (bottom).** The γ-proteobacterium *P. putida* KT2440 (top) is shown in phase contrast (left) and cyan channel (right). When imaged with a cyan filter (λ_{ex} of 436 nm; λ_{em} of 480 nm), pyoverdine is visible in the periplasmic space. Scale bar: 2 µM. The image was taken with a Leica DMi8 inverted microscope with a Leica DFC365 FX camera. The pathogenic γ-proteobacterium *P. aeruginosa* was imaged using a transmission electron microscope. Four polar flagella are visible. The bottom figure was taken from *Brock Mikrobiologie* Version 15 [8]. The montage was created using Fiji [20].

The genome of *P. putida* KT2440 includes 5729 annotated genes [21] and 5564 predicted protein-coding genes with a total length of 6,181,873 base pairs (bp) [22]. The genome sequence was first published in 2002 [23] and re-annotated in 2016 [24]. *P. putida* has over 85% of its coding regions in common with *Pseudomonas aeruginosa*, yet it lacks crucial virulence factors, such as exotoxins [25]. The latter bacterium is a human pathogen that causes hospital infections. It is a growing threat to humans, animals, and plants and is associated with high mortality rates [19].

Historically, *P. putida* KT2440 was first isolated in 1960 as *Pseudomonas arvilla* mt-2 harboring a TOL plasmid. Later, a modified version of this strain was classified as

Pseudomonas putida mt-2 [26], whose plasmid-free derivative strain *Pseudomonas putida* KT2440 was generated and is now used as a biosafety strain. *P. putida* strains have a versatile metabolism and a high intrinsic tolerance to various toxic substances, such as xenobiotics and organic solvents. Therefore, they can quickly adapt to new habitats and colonize new niches [27, 28]. Pollutants, such as dioxin derivatives fluorene and carbazole or polycyclic aromatic hydrocarbons (PAH), are highly toxic and mutagenic to living organisms in nature. *P. putida* strains are therefore frequently used for bioremediation, as they can degrade the just mentioned pollutants at low costs [28, 29]. *P. putida* is also used as a biocatalyst for the industrial production of various compounds, such as polyketides, non-ribosomal peptides, aromatic and non-aromatic compounds, terpenoids for the flavor industry, and promising biosurfactants such as rhamnolipids and putisolvins. Furthermore, it can produce natural substances and encodes pathways for synthesizing different secondary metabolites, such as the bio-polyesters polyhydroxyalkanoates (PHA) [17, 28, 30]. In addition, *P. putida* was recently reported to have bacteriocins such as putidacin L1 [31] and other antibiotic and anticancer compounds.

Despite its classification as a plant colonizing bacterium, strains of *Pseudomonas putida* have become increasingly resistant to antibiotics of various classes, such as β-lactams and carbapenems (Figure 2). Recently, they are causing more and more infections, especially in immunocompromised patients, even though they are less virulent than *P. aeruginosa* strains. *P. putida* strains tend to acquire multidrug resistance, especially through horizontal gene transfer using plasmids, such as pPC9, and are increasingly found in isolates from patients [27].



Imipenem **Penicillin**

Figure 2: **Antibiotics *Pseudomonas* species are becoming more resistant.** The carbapenem imipenem (left) and penicillin (right), belonging to the group of β-lactam antibiotics, are only examples of the various antibiotics *Pseudomonas* species are becoming more resistant in recent years. Reasons for that are various, including overexpression of efflux systems and the presence of β-lactamases to degrade these compounds [3, 32, 33]. Structures PubChem IDs: Imipenem: 104838*;* Penicillin G: 5904. Structures were modified using ChemDraw Version 19.1.1.21.

Therefore, MDR-*P. putida* is becoming an opportunistic pathogen for humans taking advantage of the already weakened condition of these patients. Another important factor is the ability of the strain to colonize different types of surfaces in hospitals, leading to nosocomial infections [34, 35]. One crucial factor for surface colonization in hospitals

or infection of patients is the availability of nutrients and the accessibility of ions such as iron. Since soluble iron ions are usually present in low concentrations, bacteria have developed different strategies for acquiring iron, depending on the conditions of colonization. One of them is the secretion of siderophores [2, 36, 37].

1.2 Iron and the need for siderophores

Iron is essential for several biological processes in humans and bacteria, including electron transport, red blood cell production, regulation of cell growth and division, and synthesis of DNA and RNA. Iron is present in enzymes involved in metabolic and respiratory reactions in microorganisms, such as Fe-S cluster-containing cytochromes and the aconitase of the tricarboxylic acid (TCA) cycle [8, 38]. Iron uptake and release are tightly regulated to prevent the formation of reactive oxygen species (ROS) by the Fenton/Haber-Weiss reaction, in which free Fe^{2+} reacts with hydrogen peroxide [38, 39]. However, not only too much iron is a problem for bacteria, but also iron deficiency: microbial growth becomes problematic when the intracellular iron concentration falls below 10^{-6} M [40]. Therefore, many bacteria produce siderophores as secondary metabolites secreted to chelate iron [39, 41, 42]. Figure 3 illustrates the different structures of siderophores found in bacteria. So far, over 500 types of siderophores have been identified in bacteria, fungi, and plants [43]. Many bacteria produce more than one type of siderophore: For example, uropathogenic forms of *E. coli* (UPEC) can produce three different siderophores optimized for iron chelation under different pH conditions: aerobactin, enterobactin, and yersiniabactin [44]. These molecules can bind to iron ions by providing an oxygen ligand for Fe^{3+} (Figure 3).

The name siderophore comes from Greek and means *iron carrier* [45]. These iron chelators are produced by bacteria, fungi, and plants [2, 5, 46] and are crucial for survival in iron-deficient environments. At the same time, they are essential for host colonization and interbacterial competition and have many different structures composed of various chemical functional groups (Figure 3) [47]. They are produced by either non-ribosomal peptide synthetases (NRPS) or polyketide synthetases (PKS), and the molecular weight of these small peptides ranges from 200 to 2000 Dalton [45, 47, 48]. In fluorescent pseudomonads, the primary siderophore is fluorescent pyoverdine (PVD) [5], which is also referred to as fluorescein or pseudobactin in the literature [47]. For *P. putida* KT2440, pyoverdine can be nicely visualized in the periplasm using fluorescence

microscopy (Figure 1, top). It is produced by many pseudomonads, such as *P. putida*, *P. aeruginosa*, *P. syringae*, and *P. fluorescens* [48].

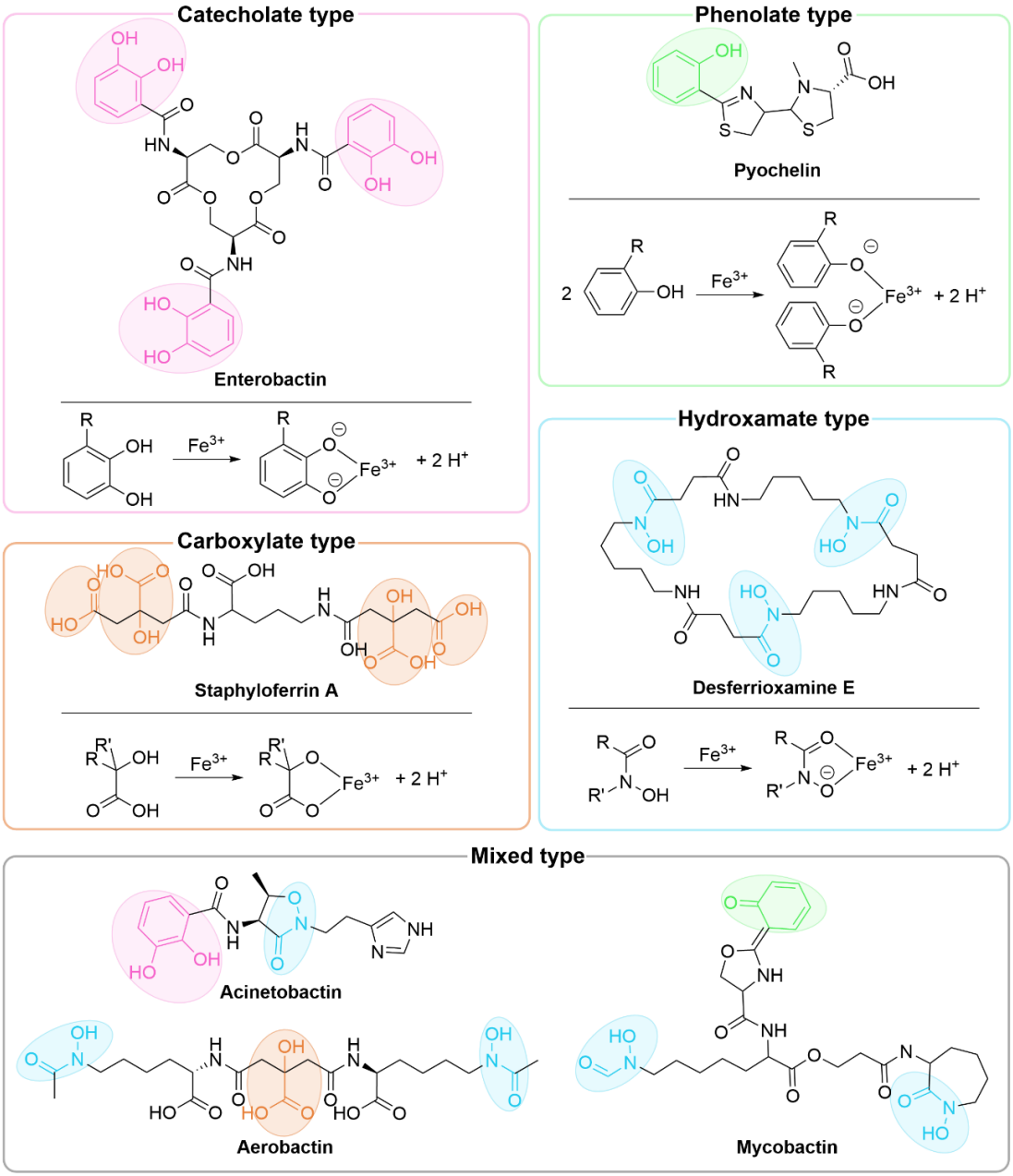


Figure 3: **Structures of siderophores produced by different microorganisms**. Groups responsible for iron chelation are shown in color. The respective modes of coordination for each class of siderophore are given below. Chemical structures were taken from Pubchem (pubchem.ncbi.nlm.nih.gov) with the following compound CIDs: enterobactin, *Escherichia coli*, 34231; staphyloferrin A, *Staphylococcus hyicus*, 3035516; acinetobactin, *Acinetobacter baumannii*, 49802052; aerobactin, *Escherichia coli*, 123762; pyochelin, *Pseudomonas aeruginosa*, 443588; desferrioxamine E, *Streptomyces parvulus*, 161532; mycobactin, *Mycobacterium tuberculosis*, 3083702. The figure was adapted and expanded from Wilson *et al.* [49] using ChemDraw Version 19.1.1.21.

In addition to primary siderophores, which have a strong affinity for iron, many pseudomonads produce secondary siderophores with lower affinity. The secondary siderophores have structural differences, including that some are derived from peptides, such as pyoverdine, while others are synthesized differently. These secondary siderophores include pyochelin, pseudomonine, ornicorrugatin and corrugatin, yersiniabactin, achromobactin, PDTC, and quinolobactin. The siderophore pyochelin is a non-fluorescent siderophore produced by some pseudomonads, including *P. putida* [2].

Siderophores form stable and soluble complexes with Fe^{3+} and exhibit an extremely high affinity of 10^{17} to 10^{43} M^{-1} [47]. Besides binding Fe^{3+} ions, pyoverdine is also present in complexes with other trivalent and divalent metal ions (Al^{3+}, Ga^{3+}, Mn^{2+}, Ni^{2+}) and boron. Other naturally occurring iron chelators produced by microorganisms are zincophores (Zn^{2+}), which chelate zinc ions, or chalcophores, that bind copper ions (Cu^{2+}) [48, 50]. Some siderophores also have antibiotic activity [50] or can act as signaling molecules [48], and sometimes they even can act as virulence factors [51]. Another essential side function of some siderophores is detoxifying heavy metals and regulating oxidative stress [50]. In addition to the production of siderophores by every single individuum, communities of pseudomonads have been described as cooperating in a complex pattern to exchange this *public good* [52] or to use siderophores produced by other species, so-called xenosiderophores. In this way, they avoid the costly production of siderophores by themselves.

The uptake of metal ion-loaded siderophores and xenosiderophores occurs via TonB-dependent mechanisms. The TonB receptor is a transmembrane protein that spans the cell membrane and is essential for transporting iron-siderophore complexes across the membrane. It is thought to utilize the gradient-generated proton motive force (*pmf*) across the cell membrane to actively transport the iron-siderophore complexes into the cell [48, 53-55]. In addition to importing xenosiderophores, pseudomonads also use heme-containing proteins as a source of iron. For this purpose, the heme group must be extracted from the originating protein, such as hemoglobin [38, 40]. To prevent pathogenic bacteria from obtaining the iron they need to grow and colonize the host, mammals have evolved proteins such as lactoferrin and transferrin that scavenge iron (Figure 4, 1). However, the affinity of siderophores for iron ions is often much higher (to the detriment of the host) than that of these host-derived proteins. The k_D value of transferrin towards trivalent iron is 10^{-22} M [40], while the affinity of siderophores can be as high as 10^{32} M^{-1} [48] (Figure 4, 2). To counteract this, mammals have evolved siderocalin, also called lipocalin, a protein with the ability to bind siderophores such as

enterobactin, bacillibactin, and carboxymycobactin and prevent them from delivering iron to pathogenic bacteria (Figure 4, 4). This strategy is part of the immune response and helps to limit the spread of infection and maintain iron homeostasis in the body [5, 40].

Human albumin has a relatively weak affinity for Fe-enterobactin, with a k_D value of only 10^{-5} M. This means that it is unable to compete effectively with the innate mechanism of Fe-enterobactin uptake by *E. coli* via the FepA receptor, which has an affinity for Fe-enterobactin of 2 x 10^{-10} M. Siderocalin, on the other hand, has a stronger affinity for Fe-enterobactin, with a k_D value of 4x10^{-10} M [40]. Unfortunately for the host, data suggest that pyoverdins, one of the two siderophores of pseudomonads, are not efficiently recognized by siderocalin [56].

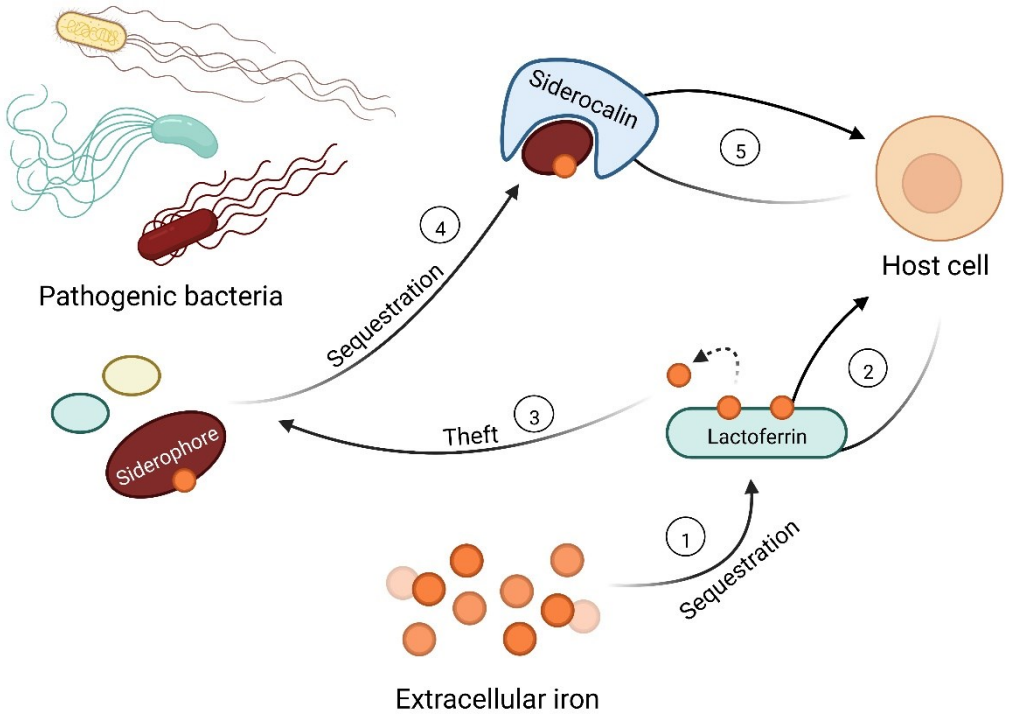


Figure 4: **Circuit of iron chelation and sequestration by mammalian and bacterial cells.** Mammalian cells secrete iron-binding lactoferrin to chelate iron from the environment. This protein harbors two iron-binding sites (1). Host cells can take up iron-loaded lactoferrin (2). To use bound iron, bacteria, such as *E. coli*, *P. aeruginosa*, and *S. enterica*, developed siderophores with a higher affinity towards Fe^{3+} and can thereby steal the iron from lactoferrin (3). Mammals evolved a counter strategy by sequestering iron-bound siderophores (4) and subsequently taking them up (5) [40]. The figure was generated based on an illustration by Wilson *et al.* [49] using BioRender.com.

1.3 Pyoverdine

Several strains of *Pseudomonas* are known to produce a fluorescent siderophore (Figure 1, cyan channel). Although these fluorescent pigments were discovered over a century ago, their importance and function in iron-deficient environments did not become apparent until the late 1970s [57]. In 1966, fluorescent pseudomonads were published for the first time, and with further discoveries, it became clear that this property is widespread in the *P. putida* group [3, 28, 58]. The *P. putida* KT2440 genome encodes 26 genes that are thought to play a role in the pyoverdine circuit, including its synthesis, regulation, and transport.

In general, pyoverdines can be characterized by three properties: (I) an acyl side chain (Figure 5, 3-amino group of the chromophore), which consists of either a malic, succinic, or glutaric acid residue or the corresponding amides. (II) a pyoverdine chromophore (Figure 5, cyan), derived from 2,3-diamino-6,7-dihydroxyquinoline. (III) a strain-specific peptide chain (Figure 5, green). It is attached to the N-terminal chromophore and contains 6-14 canonical and non-canonical amino acids. The acyl side chain is strain specific, whereas the chromophore is highly conserved among the whole *Pseudomonas* species [1, 3, 48]. Therefore - pyoverdine produced by a single strain - has an identical peptide backbone but variable side chains, whereas pyoverdine produced by closely related strains may also have variable peptide backbones [5].

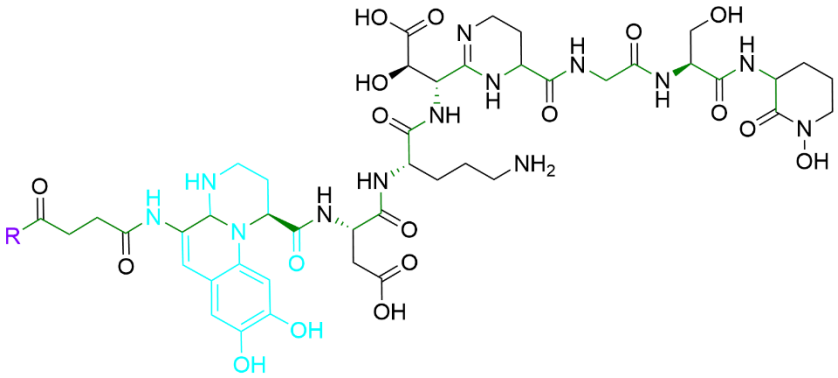


Figure 5: **Structure of pyoverdine from *P. putida* KT2440**. The dihydroxyquinoline chromophore in its carboxylic acid form is highlighted in cyan, whereas green represents the peptide backbone. The position of the variable R-group is indicated by the purple R. Depending on whether the stated residue (R) is a primary amine or a hydroxyl group, one of the two forms of pyoverdine, namely G4R A or G4R, is present [1]. The structure was taken from PubChem with the corresponding CID 102426994 and modified in ChemDraw Version 19.1.1.21.

Pyoverdine synthesis genes are primarily located in a defined region inside the genome, also called the *pyoverdine region*. For *P. aeruginosa*, this region has an impressive size of 103 kilobases [3]. Pyoverdine synthesis and maturation occur in different cellular compartments: First, non-ribosomal peptide synthetases (NRPS)

generate the non-fluorescent precursor in the cytoplasm. PfrI (PvdS in *P. aeruginosa*) and PP_4208 (FpvI) regulate this process [59, 60]. The ABC -transporter pvdE (PP_4216), present in the cytoplasmic membrane, is responsible for transporting a non-fluorescent pyoverdine precursor into the periplasmic space [3, 48]. There, ongoing maturation processes result in the cyclization of the chromophore, creating the fluorescent form of pyoverdine (cyan, Figure 5). In the next step, it is bound to the peptide chain (green, Figure 5), which in the case of pyoverdine G4R, has been analyzed by various techniques, including FAB-MS, NMR spectroscopy [61], NRPS prediction, and isoelectric focusing (IEF) [62]. The specific side chain of *P. putida* KT2440 was confirmed to be Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-OHOrn (Figure 5) [1, 61-64]. The cyclic character of pyoverdine and the amino acid composition of D- and L-amino acids show similarity towards antibiotics produced by some Gram-positive species [3]. Siderophores, including pyoverdine produced by *Pseudomonas* strains, can be produced in large quantities and have practical applications in research and biotechnology: one example is the use of IEF to identify and classify bacterial species based on their characteristic structure and side groups within the *Pseudomonas* group, a technique called "sideroptyping" [2, 3]. Another example is bioremediation using pyoverdine as a biosensor to detect the carcinogenic furazolidone in the environment. Pyoverdine can quench the fluorescence of furazolidone, providing a rapid and inexpensive method for detecting this pollutant [65].

The siderophore can either be secreted or remains in the periplasm, where pyoverdine can be detected by its absorbance at 400 nm [66, 67]. Upon uptake into the periplasm, pyoverdines are not metabolized or modified. Instead, ferric iron is reduced in the periplasm to generate apo-pyoverdine and free Fe^{2+} ions, which are taken up by the ABC transporter FpvDE [3, 48]. In order to control the availability of iron and siderophores simultaneously, genes involved in this process need to be tightly regulated.

1.4 Regulatory mechanisms of siderophore synthesis and transport in bacteria

Enzymes and transport systems that enable siderophore biosynthesis, secretion, uptake, and release of iron must be tightly controlled in every organism. In bacteria, gene regulation of siderophore utilization and iron homeostasis occurs primarily at the transcriptional level. The ferric uptake regulator Fur directly or indirectly regulates many genes for iron uptake and storage [3]. Other regulatory elements, such as extracytoplasmic σ-factors (σ^{ECF}) and their respective anti-sigma factors, additionally

play an essential role [45, 48, 55]. Bacterial σ-factors reversibly bind to DNA-dependent RNA polymerase (RNAP) in the bacterial cell (Figure 6), which is necessary for regulating gene expression in bacteria. Thereby they assist in adaptation processes to changing environmental conditions [68]. These regulatory factors act together to control several genes simultaneously.

In *E. coli* and many other bacteria, the Fur protein regulates the expression of over 90 genes involved in iron metabolism. Fur is a dimer of 17-kDa subunits and functions as a positive repressor that represses transcription in the presence of iron and enables transcription in the absence of this ion (Figure 6). The FecI of *E. coli* and the PvdS proteins of *P. aeruginosa* (PA2426) were identified as members of a group of σ^{ECF} factors involved in iron metabolism. The homolog of PvdS in *P. putida* is PfrI (PP_4244), with 84% identity at the protein level. This group has been termed the "iron starvation sigma factors" because they regulate several iron-related processes [36, 60]. PvdS and PfrI are responsible for the transcriptional activation of genes for the biosynthesis of pyoverdine and pseudobactin in *Pseudomonas*.

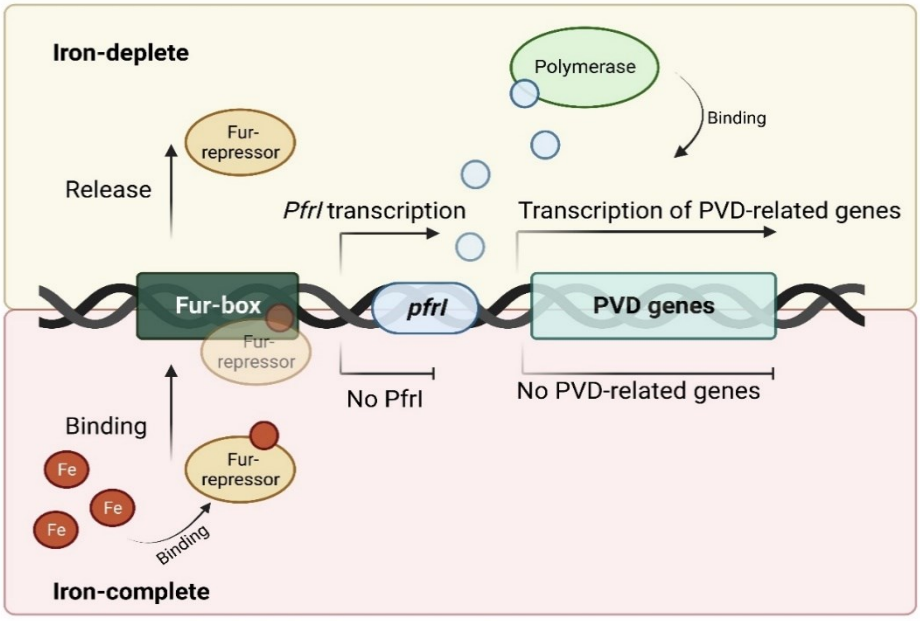


Figure 6: **Simplified schematic representation of iron-dependent gene expression.** When sufficient iron is present (red area), the Fur repressor protein binds to iron (Fe^{2+}) and prevents transcription of the σ-factor PfrI (PP_4244) by binding to the Fur-box. However, at low iron concentrations (yellow area), the Fur repressor is no longer bound to iron and dissociates from the Fur-box, initiating PfrI transcription. PfrI forms a complex with the core RNA polymerase (CRNAP), and pyoverdine-related genes are transcribed. The figure is based on a publication by Leoni *et al.* [60] and was generated using BioRender.com.

The PvdS/PfrI expression process is directly controlled through iron availability sensed by the Fur repressor [60] (Figure 6). It has been reported that other factors than iron can also induce PvdS-related gene expression, such as population density and copper starvation. Besides the control of siderophore synthesis by PvdS and PfrI, the anti- σ-factor FpvR (PP_3555, PA_2388) can upregulate the synthesis processes if pyoverdine is recognized by its outer membrane receptor FpvA [69].

In addition to the production of genes for pyoverdine synthesis, transporting this siderophore is another essential step for iron acquisition. Siderophores are secreted in an energy-consuming process through tripartite efflux systems that span all three compartments of the Gram-negative cell (inner membrane, periplasm, and outer membrane). These efflux pumps belong to different protein superfamilies depending on the type of siderophore they transport [45] (Chapter 1.5). In general, efflux systems that are interdependent from each other are tightly regulated so that efflux pumps are produced at controlled levels. As a result, the expression of additional pumps may be affected by the deletion or suppression of another efflux pump [70]. The operation of efflux systems requires high energy [30]. Depending on the given metabolic conditions, this can be between 10 and 60 % of the adenosine triphosphate (ATP) in a bacterial or human cell [71]. Therefore, local and global activators and repressors influence the control of tripartite efflux systems at the transcriptional level in response to various environmental signals, including antibiotics, iron, and non-antibiotic substances, such as antidepressants and biocides [72].

Four families of transcriptional regulators involved in regulating RND efflux pumps, one type of tripartite efflux system in *P. aeruginosa*, have been described so far: the LysR, MarR, AraC, and TetR classes of regulators. For example, members of the TetR family locally regulate the expression of tripartite efflux pumps by binding potential substrates of the pump (ligand-repressor interaction) and subsequently enabling the transcription of components of the TetA transport machinery. One member of the TetR family, TtgR (PP_1387), has been shown to actively bind different molecules, which indicates a broad substrate spectrum of the corresponding pump TtgABC [73]. Another strategy of regulation is by two-component systems (TCS). For *P. aeruginosa*, five TCS have been identified as critical regulatory mechanisms for controlling RND efflux pumps [73, 74]. As another example, efflux pump expression can also be regulated post-transcriptionally, which was recently reported for the efflux system AcrAB-TolC [32, 72]. This overall tight regulation of the expression of complete tripartite efflux systems may be necessary because overexpression can lead to toxicity [33].

1.5 Tripartite efflux pumps

There are two general types of transport processes across bacterial membranes: passive and active. Passive transport, which includes passive and facilitated diffusion, moves molecules across the membrane following a concentration gradient. Active transport requires energy and can move molecules against their concentration gradient. While passive diffusion can occur directly through the membrane's lipid bilayer, active and facilitated diffusion require transport proteins [75]. The Gram-negative bacterial cell envelope consists of three main barriers, the outer membrane (OM), the aqueous periplasm (PP), and the inner membrane (IM). The OM is a barrier for various substances by abolishing diffusion through the membrane. To simultaneously enable transport across all three compartments, Gram-negative bacteria have developed tripartite efflux systems, mainly organized in operons, which can transport substances from the cytoplasmic and the periplasmic space into the environment. The biology and natural defense of bacteria depend on those efflux pumps (Figure 7) [73, 76].

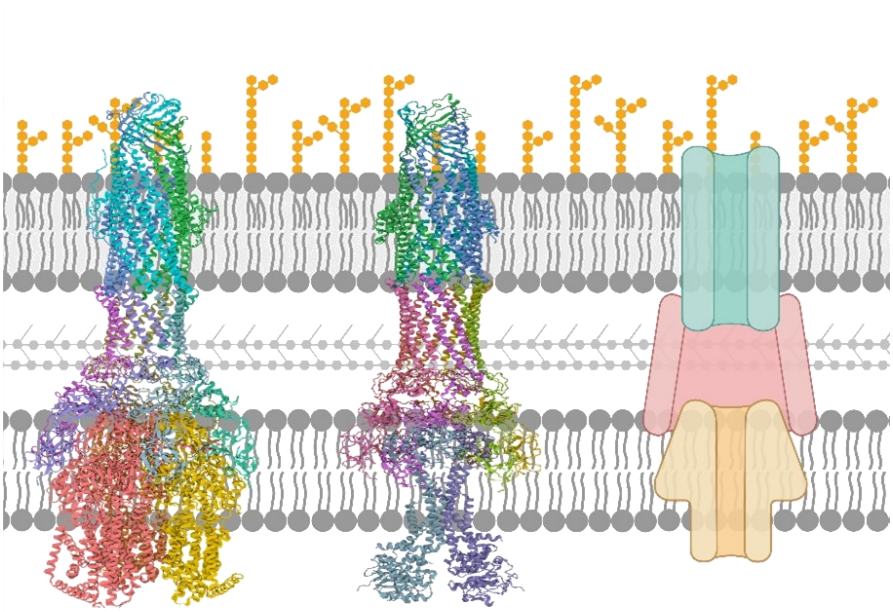


Figure 7: **Schematic representation and crystal structures of two types of tripartite efflux systems.** From left to right: Crystal structures of the RND type efflux system MexAB-OprN from *Pseudomonas aeruginosa* PAO1 (PDB ID:6IOK) and the ABC type efflux system MacAB-TolC from *Escherichia coli* K12(PDB ID: 5NIL). Right side: schematic representation of a tripartite efflux system. Here, the outer membrane porin (OMP) is highlighted in green, the adapter protein (MFP) in red, and the inner membrane protein (IMP) in orange. The main structural difference between the two systems shown is the trimeric inner membrane protein for the RND system and the dimeric inner membrane protein for the ABC system. The figure was generated using BioRender.com.

Efflux pumps have been classified into six families based on the amino acid sequence, energy source required for export, and substrate specificity [32]. These systems use the proton motive force (*pmf*), sodium-ion motive force (*smf*), or ATP hydrolysis to energize transport. Transporters that work with *pmf* belong to either the resistance-nodulation-cell division group (RND), the major facilitator superfamily (MFS), the proteobacterial antimicrobial compound efflux (PACE), or the small multidrug resistance (SMR) family. The multidrug and toxin extrusion (MATE) family uses either the *pmf* or the *smf*. In contrast, the only family that uses ATP hydrolysis for transport is the ATP-binding cassette (ABC) family.

Examples of an RND (left) and an ABC (right) transporter are shown in Figure 7. Together, these two families form one of the groups of efflux pumps responsible for antibiotic resistance by transporting different antibiotics and antimicrobials out of the cell and into the environment [32, 73, 77]. Only the transporter groups RND, ABC, and MFS can form tripartite complexes, while the families PACE, MATE, and SMR are localized in the inner membrane as independent proteins. Therefore, these families transport substrates from the cytoplasm to the periplasmic space rather than the environment [78-80]. Currently, 290 ABC, 40 RND, 88 MFS, two SMR, three PACE, and two MATE transporters are annotated in the genome of *P. putida* KT2440 (membranetransport.org).

As the name implies, tripartite efflux systems have a common feature: the composition of three parts (Figure 7). The inner membrane protein (IMP) acts as the motor, using either ATP (ABC) or a proton gradient (RND) to enable transport. The composition of the inner membrane protein is also characteristic of the number of transmembrane helices (TMH) each protein has: SMR-class transporters have only four, and RND-class transporters have 12. MFS-class transporters have either 12 or 14 TM helices [79]. The number of TMH for ABC transporters varies between 10 to 14 for the whole IMP [71]. The molecules are discharged through an outer membrane porin (OMP), which in both cases is located in the outer membrane of the Gram-negative leaflet. The two parts of the inner and outer membrane are connected by a periplasmic adapter or membrane fusion protein (PAP/MFP), which is essential for the interaction and functionality of the efflux pump [29, 30, 33, 81]. Especially the periplasmic part of the inner membrane protein of RND pumps has been described as crucial for substrate recognition [79].

ABC and RND transporters are not limited to bacteria but are also present in archaea and eukaryotes [8, 82, 83]. Especially in Gram-negative bacteria, they have broad and overlapping substrate specificities. This property confers a so-called *intrinsic resistance* to bacteria, meaning a substance is excreted immediately after passing through the

membrane by transporters with a broad substrate spectrum and constitutive expression [78]. In addition to transporting antibiotics, efflux pumps of the transporter classes ABC and RND are involved in several other critical processes, including the secretion of virulence factors, quorum sensing molecules, fatty acids, harmful lipids, organic solvents, detergents, iron chelators, and toxins. They are also essential for defending bacteria against natural compounds from the colonized host [73, 78, 84, 85]. This very diverse substrate spectrum exhibited by all tripartite efflux systems is probably related to the fact that these pumps evolved long before the age of antibiotics, and their emergence was an ancient biological event [86]. In *E. coli*, over 550 transporters have been identified, and about 5 to 10% of its genome encodes genes involved in transport processes, for instance, ABC transporters [71].

1.5.1 The ABC system PvdRT-OpmQ is a proposed pyoverdine transporter

The group of ABC transporters is named after their energy source ATP (ATP binding cassette). ABC transporters are essential in importing and exporting substances and intracellular signaling processes in eukaryotes [71, 87]. They can transport substances of different classes, from small molecules such as ions to antibiotics such as aminoglycosides, quinolones, and tetracycline. In addition, they can also transport larger substances such as dyes, lipids, and polysaccharides [33, 79, 87]. More than 200 different ABC transporters have been identified in bacteria so far [8], and the genome of *E. coli* encodes for more than 79 different ABC transporters [88].

In contrast, the human genome encodes about 50 of them. Cholesterol and lipid transport are some examples carried out by members of the seven subfamilies of human ABC transporters. Mutations in these proteins have been associated with various diseases [71, 89]. In eukaryotes, the ABC transporters are the primary efflux system, whereas, in prokaryotes, multiple efflux systems are involved, as described in 1.5 [79].

ABC transporters have a distinctive architecture, including two nucleotide-binding domains (NBD) located in the cytoplasm, and two transmembrane domains (TMD), anchoring the transporter in the inner membrane. Whereas the TMDs are prone to high diversity among the different types of ABC transporters present, the ABC subunit, on the other hand, is mainly conserved among organisms. The ABC subunit is further divided into a catalytical and an α-helical domain. The catalytical domain contains both a Walker A and B motif, whereas the α-helical domain contains the signature motif LSGGQ, which is characteristic of ABC transporters. ABC transporters are present as dimers, where the binding site for ATP is present in the middle between both monomers - depending on the

sort of ABC transporter, the TMD range from 5 to 7 TM helices, resulting in 10 to 14 TM helices per dimer [33, 71].

Substrates of bacterial ABC exporters are not only antibiotics, even though they were discovered as drug transporters. They are critical for transporting dyes, ionophoric peptides, lipids, and steroids. On the other hand, the substrates of ABC importers vary significantly in size and chemical nature and can include oligopeptides, oligosaccharides, small ions, and different types of molecules [71]. Besides the transport of antibiotics and other compounds, ABC transporters have been described as participators in the transport of siderophores. One system has attracted particular interest since its involvement in the transport of the newly synthesized and recycled siderophore pyoverdine was already proposed for *P. putida* and *P. aeruginosa* in the past: The PvdRT-OpmQ system, which is a ubiquitous ABC transport system in many pseudomonads [12, 67, 90].

Homologs of it, such as the MacAB system from *E. coli* (*Ec*MacAB) [91], have been characterized in the past for antibiotic resistance spectra, their crystal structure, ATPase function, and involvement in the transport of diverse substrates [92, 93]. *P. aeruginosa* appears to have PvdRT-OpmQ as a main pyoverdine transport system [12, 67], whereas, for *P. putida* KT2440, two systems have been described. One of them is the homologous system PvdRT-OpmQ (identity >60% to *P. aeruginosa*) [90]. Nevertheless, upon deletion of PvdRT-OpmQ in *P. aeruginosa* and *P. putida* KT2440, pyoverdine secretion could never be entirely abolished. The secretion was reduced to 50-60%, and at the same time, an accumulation of the siderophore in the periplasmic space became visible [12, 48, 67, 90]. In addition to the PvdRT-OpmQ system (*Pp*PvdRT-OpmQ), another system was identified in *P. putida* KT2440: the RND efflux system MdtABC-OpmB (*Pp*MdtABC-OpmB). Since a double deletion mutant for the *Pp*PvdRT-OpmQ and the *Pp*MdtABC-OpmB systems is still able to secrete the siderophore, the mechanism of secretion is not yet fully understood, but the participation of other efflux systems seems likely [90]. Since tripartite efflux systems represent systems with a huge substrate diversity [94], particular emphasis was placed on RND transporters besides ABC efflux systems.

1.5.2 The RND type efflux pumps TtgABC and ParXY and their contribution to efflux in *P. putida* KT2440

Several microbial organisms express multiple efflux pumps from the resistance-nodulation- cell division group. Some systems can be expressed constitutively, while others are activated only when a substrate is present (Chapter 1.4) [72, 78]. RND systems are responsible for resistance to different types of substrates with amphiphilic

(HAE) or hydrophobic (HME) characteristics [95]. These substrates include aromatic hydrocarbons [16, 30], antibiotics [30, 33, 96], and natural bipyridyl derivatives [85]. In addition, RND efflux pumps transport organic solvents [16, 97] and quorum-sensing signals [78, 98]. They can also contribute to environmental adaptation [38] and pathogenesis [78]. Inactivation of only one RND transporter (e.g., by mutation) can render a resistant strain highly sensitive to different classes of antibiotics [78].

The genome of *P. putida* encodes at least 14 RND efflux systems, of which the TtgABC system is the only one that - by experimental evidence - confers multidrug resistance [16]. Several RND systems are encoded as operons containing all three parts of the tripartite efflux system, but not all of them. For example, several Mex systems of *P. aeruginosa*, such as MexXY and MexJK, exploit multiple outer membrane proteins while not encoding an OMP gene inside their own operon [96]. For RND systems of *E. coli*, TolC functions as the main outer membrane protein, interacting with at least eight other efflux systems [99]. In *E. coli*, three RND systems are involved in enterobactin transport: AcrAB, AcrCD, and MdtABC, a homolog of the MdtABC-OpmB system of *P. putida* KT2440. These two systems share a sequence identity of more than 52% [90, 100]. During this thesis, two RND systems from *P. putida* KT2440 were further investigated: the TtgABC (*Pp*TtgABC) and ParXY (*Pp*ParXY) systems. *Pp*TtgABC (Chapter 3) and *Pp*ParXY (Chapter 4) were analyzed for their involvement in pyoverdine secretion and other potential substrates they transport.

The TtgABC system is one of the main RND efflux systems of pseudomonads [97], which is why it can be found in *P. putida* B6-2 [29], *P. putida* KT2440 [101, 102], *P. putida* DOT-T1E [29, 97], *P. aeruginosa* [85] but also in other human pathogens such as *A. baumannii* as the homologous AdeAB system [80]. The closest homolog to the *Pp*TtgABC system found in *E. coli* is the AcrAB-TolC efflux system (string-db.org) [30]. *Pp*TtgABC is organized as an operon, with genes PP_1386/85/84 encoding the MFP (TtgA), the IMP (TtgB), and the OMP (TtgC). The name of the group of *ttg*-related genes derives from the ability of these systems to excrete toluene (toluene tolerance gene), the substrate initially discovered for this class. Several other efflux systems have been identified within the Ttg group, including TtgABC, TtgDEF, and TtgGHI. All three systems transport solvents in *P. putida* strains [98, 103]. Additionally, the *Pp*TtgABC is responsible for the efflux of toluene, flavonoids, and antibiotics such as chloramphenicol, carbenicillin, tetracycline, erythromycin, and nalidixic acid [16, 97]. The other system, TtgDEF, additionally transports styrene and toluene, while TtgGHI transports several classes of antibiotics, including chloramphenicol, ampicillin, and tetracycline [29]. In *P. putida* DOT-T1E, the TtgGHI system is encoded on a plasmid (pGRT1) [16] from where it is

expressed constitutively at high levels, independent of induction by an antibiotic [97]. Since the TtgABC system represents a fundamental efflux system in *P. putida* KT2440, it was initially investigated for its involvement in siderophore secretion in this strain. Later, the participation of this system in the secretion of the iron chelator 2,2' bipyridyl (Bip) and natural derivates of this compound, as well as bile salts and deoxycholate, could be shown [85].

The second further investigated RND system ParXY is a just newly discovered system of *P. putida* KT2440. It was first identified in a multidrug-resistant version of the strain. The MexCD (PP_2817/18) system of *P. aeruginosa* is the closest related system with more than 50% similarity [16]. Mex systems are widely distributed among different Gram-negative bacteria, and they do come with several orthologous systems, which is why MexCD is not the only Mex system in *P. putida* strains. Among them, we can also find the MexEF (PP_3425/26) and different transcriptional regulators, such as MexS (PP_2827) [102], which tightly control the expression of these systems. Since they all show a very high similarity, the investigation and complete elucidation of the Mex homologs is quite difficult. The MexAB system of *P. aeruginosa* PAO1, initially identified while searching for siderophore secretion systems [104], represents one of the first systems described as possible pyoverdine efflux pump in this organism [51]. Its identification and analysis indicated that this system transports a variety of substrates, and besides that, its overexpression leads to multidrug resistance [33]. Indeed, upon sequence alignment of both systems, the AcrAB system of *E. coli* shows a comparable identity to the ParXY system of *P. putida* KT2440 (protein identity MFP 46%) as the MexCD of *P. aeruginosa* PAO1. As mentioned above, the two Acr systems of *E. coli* are evidenced in the secretion of enterobactin [100]. The *Pp*ParXY system is so far described to be primarily involved in transporting aminoglycosides and was named according to this function (*putida* aminoglycoside resistance) [16].

Even though all three further investigated systems described above, *Pp*PvdRT-OpmQ, *Pp*TtgABC, and *Pp*ParXY, belong to different classes of transporters, they have their genetical organization in common. This characteristic is shared among most tripartite efflux systems identified so far [94].

1.6 Genetic organization and regulation of tripartite efflux pumps

Genes coding for tripartite efflux systems are usually allocated in operons and have, in many cases, all three components together to connect the IM and OM of a Gram-negative cell (Figure 7). Figure 8 shows the representative organization of respective

systems relevant for this thesis, including the efflux system components and the regulatory elements. Regulatory elements are essential since the expression of efflux systems varies depending on the growth phase of the microbe [95, 105]. Such regulatory elements are either close to the respective system or far away in the genome [16].

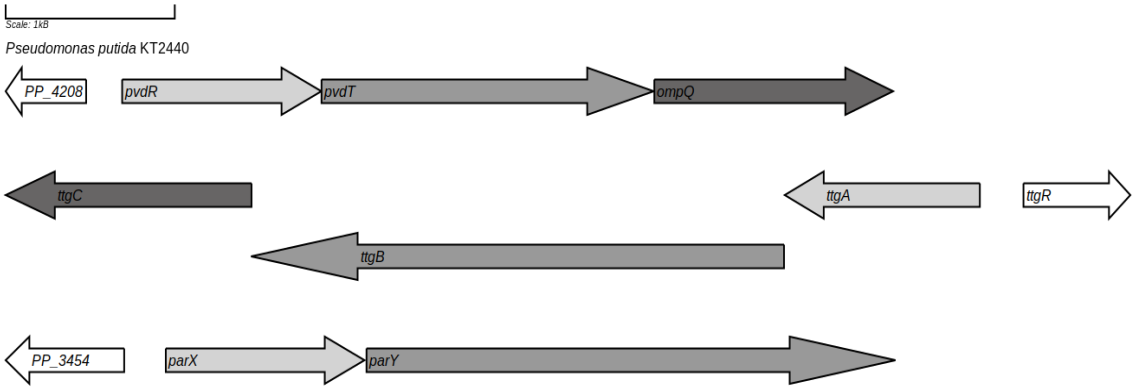


Figure 8: **Genetic allocation of the three investigated systems PvdRT-OpmQ, TtgABC, and ParXY in *P. putida* KT2440.** The genes for the inner membrane protein (IMP) are highlighted in light grey, the genes for the adapter protein (MFP) in medium grey, and the outer membrane protein (OMP) in dark grey. The MexAB/AcrAB system does not have a neighboring outer membrane protein. Adjoining regulatory elements are highlighted in white. The figure was generated using the genegraphics tool [106] using the accession numbers WP_010954964.1, WP_010952493.1, and WP_010954322.1, respectively.

All three systems (described in 1.5.1 and 1.5.2) show a similar structure, where at least IM and MFP are arranged in a joint operon (light/dark grey, Figure 8), *Pp*PvdRT-OpmQ and *Pp*TtgABC are organized as operons, with all three genes encoding the MFP, the IMP, and the OMP. The *Pp*ParXY only encodes an operon with MFP and IMP. All three systems have a regulatory element upstream of the genes encoding transporter components (white, Figure 8): PP_4208 (FpvI) is an σ^{ECF} factor, together with its corresponding anti σ-factor PP_3555 (FpvR) responsible for iron acquisition [107], *ttgR* (PP_1387) has been described as a repressor with antibiotic-inducible activity [97], and PP_3454 is annotated as a response regulator upstream of the two-component system PP_3454/53. This particular TCS (PP_3453/54) has been mentioned in the literature, but its regulatory function needs to be clarified [16]. The *Pp*TtgR repressor is a transcriptional regulator responsible for downregulating the TtgABC system under certain conditions (Chapter 1.4). The regulator *ttgR* and the *ttgABC* expression are tightly linked to antibiotic dosage, wherein the TtgR repressor binds the intergenic region between the *ttgR* and the *ttgA* gene and gets released upon the presence of hydrophobic antibiotics such as chloramphenicol [97]. In MDR isolates of *P. putida*, the *ttgR* gene is

mutated, causing an upregulation of the TtgABC system [16]. All three regulatory elements upstream of the respective systems presented in Figure 8 are in accordance with the regulatory elements described in 1.4.

The outer membrane protein is the last gene in the operon (dark grey, Figure 8), whereas the adapter protein is always the first (light grey). In the case of the *Pp*ParXY system (PP_3455 – PP_3456), the outer membrane protein is missing (Figure 8). Therefore, this system is thought to interact with several outer membrane proteins of *P. putida* KT2440, as shown for other RND transporters, such as MexXY of *P. aeruginosa* [96]. Nevertheless, TtgC was proposed as the main outer membrane protein, but biochemical data for the interaction of *Pp*ParXY-TtgC is lacking [16]. Compared to other enterobacteria, for instance, in *Salmonella ssp.*, the interaction of the OMP TolC was described for at least 7 MFP so far [108]. Homologs of TolC in *P. putida* KT2440 are present as the encoded genes PP_2558, PP_4519, PP_1798, and PP_4923. Whether these porins are interacting partners of the ParXY system is unknown.

Many tripartite efflux pumps have been characterized structurally and for their substrate specificity, their involvement in the transport and secretion of siderophores needs to be better understood. Several different transporters were investigated to understand the molecular mechanism of substrate binding and their involvement in siderophore transport. All of them belong to either the ABC or RND class. Several systems partly mediate the export of pyoverdine by pseudomonads, the most prominent being the PvdRT-OpmQ system [12, 43, 48, 67, 90]. The contribution of the MexAB system has also been discussed, but the literature is conflicting on the role of this pump [67, 104, 109]. The type-6 secretion system (T6SS) is also explicitly considered [41]. Bacteria are thought to have many siderophores export systems. Therefore, it has been challenging to characterize each system in detail up to this point [51, 67] since the deletion of one system never entirely stopped the secretion of pyoverdine [48, 67, 90].

1.7 Aim of this thesis

Even though parts of the secretion mechanism for pyoverdine efflux, uptake, and recycling are investigated, these findings only represent parts of a close interplay between many processes [12, 48, 67, 90]. Many other contributors to this circuit have been proposed in the past [41, 104], but not all of them could be finally confirmed. This thesis employed two distinct approaches to study the mechanism of siderophore secretion. The first approach focused on examining the biochemical interaction between the siderophore and its proposed transporter, aiming to confirm the *Pp*PvdRT-OpmQ system as a pyoverdine transporter and better understand its function - the second approach aimed to determine the involvement of other efflux systems in this process.

2. The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization

Nicola Victoria Stein, Michelle Eder, Sophie Brameyer, Serena Schwenkert and Heinrich Jung (2023). The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization. *FEBS Lett.* 597, 1403-1414, DOI: 10.1002/1873-3468.14601

Supplemental information Chapter 2

Nicola Victoria Stein, Michelle Eder, Sophie Brameyer, Serena Schwenkert and Heinrich Jung (2023). **The ABC transporter family efflux pump PvdRT-OpmQ of *Pseudomonas putida* KT2440: purification and initial characterization**. *FEBS Lett.* 597, 1403-1414, DOI: 10.1002/1873-3468.14601

3. Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440

Tania Henríquez, Nicola Victoria Stein and Heinrich Jung (2020). Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440. *Front. Microbiol.* 11, 1974, DOI: 10.3389/fmicb.2020.01974

Supplemental information Chapter 3

Tania Henríquez, Nicola Victoria Stein and Heinrich Jung (2020). **Resistance to bipyridyls mediated by the TtgABC efflux system in *Pseudomonas putida* KT2440.** *Front. Microbiol.* 11, 1974, DOI: 10.3389/fmicb.2020.01974

4. The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440

Nicola Victoria Stein, Michelle Eder, Fabienne Burr, Sarah Stoss, Lorenz Holzner, Hans-Henning Kunz and Heinrich Jung. The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440. *Microbiology Spectrum*, DOI: 10.1128/spectrum.02300-23

Supplemental information Chapter 4

Nicola Victoria Stein, Michelle Eder, Fabienne Burr, Sarah Stoss, Lorenz Holzner, Hans-Henning Kunz and Heinrich Jung. **The RND efflux system ParXY affects pyoverdine secretion in *Pseudomonas putida* KT2440.** *Microbiology Spectrum*, DOI*:* 10.1128/spectrum.02300-23

5. Concluding discussion

Bacteria have evolved different mechanisms to transport substances and resist antibiotics. One of these mechanisms is transport through tripartite efflux systems (Figure 7), which play an essential role in this resistance [77, 110]. Bacteria express up to 20 different efflux pumps that transport molecules, including antibiotics, solvents, bile acids, and siderophores [78, 98]. Despite decades of research, our knowledge of these systems and their original role is still limited. Technological advances in recent decades, including cryo-electron microscopy and modern analytical tools, have improved the ability of researchers to study efflux pumps [32, 111, 112]. Nevertheless, for many proteins, little information is known about the substrate spectra [110] and the original purpose of this pump [113]. The same is true for studying the regulatory mechanisms that control their expression [80].

Identification of systems involved in pyoverdine secretion is complex: even double deletion of two identified systems in *P. putida* KT2440 does not entirely abolish secretion of the siderophore since a double deletion mutant (Δ*pvdRT-opmQ* Δ*mdtA*) does still secrete 34% of WT pyoverdine levels [90]. In addition, many tripartite efflux systems in a strain have overlapping substrate spectra, leading to cross-resistance by activation of other systems upon deletion of one [95, 110]. Identification requires not only the deletion of new systems but also combinatorial deletions and bioinformatic investigations to identify possible transporters that can replace missing systems [113]. The next crucial step in understanding tripartite efflux systems is their purification and biochemical characterization. The regulatory proteins of these systems, such as TetR, are relatively easy to purify due to their cytosolic localization and solubility [74]. However, the membrane proteins that make up these systems are challenging because overexpression of respective genes is difficult and can cause toxicity, formation of aggregates, or disruption of the intact membrane topology [30, 79].

The data presented in this thesis contribute to existing research on tripartite efflux systems by identifying novel pyoverdine transport systems (Chapter 4) and providing biochemical evidence for the interaction between the putative pyoverdine transporter PvdRT-OpmQ and the siderophore of *Pseudomonas putida* KT2440 (Chapter 2). Finally, the role of the multidrug transport system *Pp*TtgABC was characterized, and a new and probably natural function of this system in the extrusion of 2,2'-bipyridyl and its derivatives was discovered (Chapter 3).

5.1 Purification of the ABC transporter PvdRT-OpmQ using its native host

The ABC transporter PvdRT-OpmQ has been described in independent studies as the putative main transport system for pyoverdine in pseudomonads [43, 90]. Nevertheless, the pyoverdine cycle, from synthesis and secretion to uptake and recycling, still needs to be thoroughly investigated. Especially the secretion mechanisms are only partially understood, and not all systems have been identified so far [43, 90]. A more detailed analysis of this transporter would ultimately confirm the idea that this transporter actively releases the siderophore pyoverdine into the environment (Chapter 2). However, purification of these proteins can be difficult as they are embedded in the hydrophobic environment of the membrane and are often expressed at low levels. In addition, the presence of other membrane proteins and lipids can make it difficult to obtain pure samples of the protein of interest. Copy numbers of membrane proteins can be low, as shown in the case of *Ec*MacAB: This system is a homologous system of *Pp*PvdRT-OpmQ in *E. coli* [51, 114], and its copy number ranges from 54 to 96 copies in MOPS minimal and complete medium, respectively [115]. Considering stoichiometry, one *E. coli* cell can survive with a minimum of 27 MacB transporters in the membrane. A low native copy number of a protein can indicate that non-physiological overexpression might be harmful to the cell, wherefore protein production using the native host of the transporter mimicking physiological conditions would be advantageous.

Several experimental steps were required to determine the specific ATPase activity of the *Pp*PvdRT-OpmQ transporter and gain insights into the physiology and substrate binding of the system (Chapter 2). The pUCP20-MCS-ANT2 plasmid, representing a synthetic system that was recently published in order to enable controlled protein production in pseudomonads, was used. The inducer of this vector system is anthranilate, an intermediate of the degradation of the amino acid tryptophan [116]. Using 1 mM of anthranilic acid yielded final amounts of 0.2 to 0.4 mg of *Pp*PvdR and *Pp*PvdT (starting from 45 mg of total membrane protein) for the two transporter components. In order to further increase the solubility and stability of the protein *Pp*PvdT during affinity chromatography, *pvdT* was cloned into pUCP20-MCS-ANT2 together with a SUMO (small ubiquitin-like protein) and a hepta-histidine tag [117]. The protein obtained could be used for downstream applications.

Furthermore, the protocol of surface plasmon resonance (SPR) was methodologically adapted to test the influence of a small substrate (~1 kDa) on the interaction of two proteins instead of testing binding to them individually (Chapter 2). After adding pyoverdine to the SPR setup, a more stable complex formation of the interaction partners

*Pp*PvdT-PvdR was visible in association and dissociation. In addition to investigating the interaction between the individual transporter components, the mass spectrometric analysis provided the information that *Pp*PvdR carries a TAT signal sequence (Chapter 2). This sequence was present after the protein purification and is unusual for transporters of this class. Whether *Pp*PvdR is indeed translocated through the TAT complex needs further investigation.

The demonstrated method of expressing genes of an ABC transporter inside the native host *P. putida* KT2440 or the derived strain KP1 may have positive implications for other research areas by improving expression strategies in bacteria for the production of, e.g., biofuels. The efflux system *Pp*TtgABC, discussed in Chapter 3, was tested for its impact on the production of improved biofuels, such as the short-chain alcohols n-butanol. Unfortunately, the expression of the *P. putida* TtgABC system in *E. coli* resulted in toxicity. In contrast, *P. putida* has already been used to produce biofuels directly from lignocellulosic biomass [30]. Using a functional system such as the pUCP20-ANT2-MCS expression system [116] in the *P. putida* KT2440 strain for our studies confirms that the choice of the expression systems is crucial for successful gene expression.

5.2 The PvdRT-OpmQ system is a putative pyoverdine transporter whose ATPase activity is dependent on the presence of the adapter protein

There is, in general, only limited biochemical evidence of the functionality of tripartite efflux systems of the ABC family, including substrate translocation and the mechanisms of ATPase function. *E. coli* MacB, a homolog of PvdT of *P. putida* (Figure 9), is, in contrast, a well-studied ATPase of an ABC transporter [83, 91, 92, 118, 119]. Unfortunately, homology does not necessarily guarantee a similar ATPase function for the homologous system in *P. putida*. Recently, *Ec*MacAB was reclassified as belonging to the subgroup seven of ABC transporters, with the ability to transport substrates from the periplasm by *mechanotransmission*. The energy needed for the transport from the periplasm to the extracellular environment is mediated by ATP hydrolysis of the NBD located in the cytoplasm [94, 120]. This transport mechanism favors the transport of substrates from the periplasmic space, as is the case for matured and recycled siderophores. To analyze the putative binding of pyoverdine to the IMP *Pp*PvdT, initial analyses were performed using the DRaCALA technique [121]. The evidence of pyoverdine binding presented in this thesis (Chapter 2) supports the idea of PvdRT-OpmQ functioning as a siderophore efflux system. Membranes prepared from a Δ*pvdRT-opmQ* Δ*mdtA* strain expressing histidine-tagged PvdT spotted on a nitrocellulose

membrane inhibited diffusion of pyoverdine and let to an accumulation of the putative ligand in the center of the membrane spot (Chapter 2). The DRaCALA assay, usually based on radioactively labeled ligands, is modified by using the characteristic light absorption of the ligand for detection. These data represent the first evidence for a potential binding of the siderophore pyoverdine to *Pp*PvdT.

For *Ec*MacB, it has been shown that ATPase activity depends on the presence of the adapter protein *Ec*MacA. Additionally, macrolide antibiotics do not increase the ATPase activity of this ABC transporter [83]. These results suggest that an MFP not only plays a role in forming a tripartite system connecting both membranes but also serves a functional purpose that enables active transport through the system, which has been previously suggested for *Ec*MacAB [83]. The presence of the adapter protein *Ec*MacA enables enhanced binding of substrates by the *Ec*MacAB complex [91]. In order to test a similar effect for the *Pp*PvdRT complex, further experiments were performed (Chapter 2).

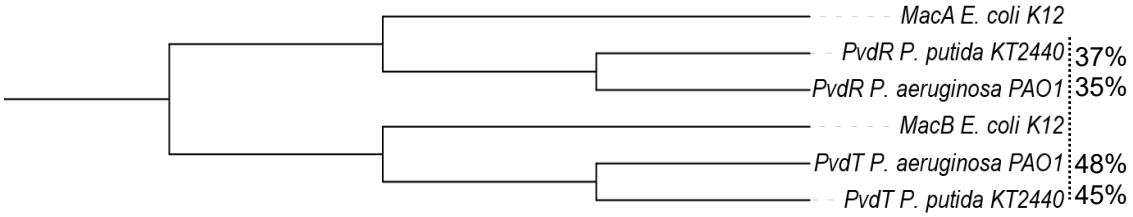


Figure 9: **Pedigree for connections between *Ec*MacB, PvdT, *Ec*MacA, and PvdR**. The percentages of protein identity are provided (on the right) when comparing the inner membrane protein *Ec*MacB to its homologs in *Pseudomonas* (PvdT) or when comparing the adapter protein *Ec*MacA to its homologs in *Pseudomonas* (PvdR), respectively. Sequences were taken from the homology search at string-db.org for *Pseudomonas putida* KT2440 [122], *Pseudomonas aeruginosa* PAO1, and *Escherichia coli* K12 MG1655. Alignment was performed using the multiple sequence alignment tool from Clustal Omega (default settings) [123, 124], and the pedigree was generated using iTOL version 6.6 [125].

The ATPase function of *Pp*PvdT was tested to evaluate further the function of the ABC transporter and the effects of pyoverdine on it (Chapter 2). The ATPase assay shows that the adapter protein *Pp*PvdR increases the $K_{M(ATP)}$ value of the complex *Pp*PvdRT for ATP. At the same time, adding the siderophore pyoverdine to the complex leads to a slight decrease in $K_{M(ATP)}$ and a lower V_{max}. As summarized in Table 1, the presence of the MFP, different detergents, or reconstitution can significantly impact enzymatic activity. For example, *Ec*MacAB had to be purified in Triton X100 because DDM inhibited ATPase activity [91]. In contrast, DDM proved to be the best detergent for the solubilization and characterization of *Pp*PvdT in our study (Chapter 2). The data presented in Chapter 2 agree with data from various biochemical reports on *Ec*MacAB [83, 91, 118]. PvdT shows basal ATPase activity even in the absence of an adaptor

protein or substrates. While *Pp*PvdT dissolved in detergents shows a similar turnover rate to *Ec*MacB in detergents [91], the K_M is more similar to *Ec*MacB in proteoliposomes [83].

In contrast, the catalytic efficiency of *Pp*PvdT alone is higher than that of *Ec*MacB in detergent but, simultaneously, becomes lower when complexed with *Pp*PvdR. In addition, pyoverdine as a substrate increases the catalytic efficiency of both *Pp*PvdT and *Pp*PvdRT, suggesting that the presence of the siderophore is favorable for the complex. The indicated effect of the siderophore as a ligand might be related to structural changes in the *Pp*PvdRT complex and supports the idea of the physiological role of the siderophore as a substrate inside the protein complex. This effect can also be seen in the SPR measurements in Chapter 2: here, incubation with pyoverdine before interaction with the immobilized adapter protein *Pp*PvdR leads to the formation of a more stable complex, which can be deduced from the increase in k_{on} and a decrease in k_{off} values. Whether pyoverdine subsequently enables a more closed conformation with controlled hydrolysis of ATP at the same rate as substrate transport needs further investigation. Techniques such as crystallizing the complex with and without substrates and FRET (Förster resonance energy transfer) could provide evidence of conformational changes [94]. Another way to test the influence of pyoverdine on binding to the transporter would be fluorescence anisotropy [126].

Table 1: Comparison of kinetic parameters for MacAB of *E. coli* and PvdRT of *P. putida* KT2440.

Protein	V_{max} [nmoles ATP mg^{-1} protein min^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]	Reference
$MacB^{TX}$	*nd*	0.91	0.17	190	[83]
$MacB^{PL}$	*nd*	2.30	0.10	43.98	
$MacAB^{PL}$	*nd*	0.38	0.78	2074	
$MacB^{D}$	8.9	0.37	0.01	*nd (27.0)*	[91]
$MacAB^{D}$	12.3	0.072	0.02	*nd (277.7)*	
$MacB^{TX}$	1820	1.74	0.07	41.5	[118]
$MacAB^{TX}$	2290	2.29	0.091	49.4	
$PvdT^{D}$	9.91	0.16	0.08	583.6	Chapter 2
$PvdRT^{D}$	69.97	2.58	0.58	214.2	
$PvdT+PVD^{D}$	6.43	0.06	0.05	955.9	
$PvdRT+PVD^{D}$	40.41	1.30	0.34	241.7	

TX, triton X-100; PL, proteoliposomes; D, DDM; *nd*, not disclosed in the publication.

Although using detergents for the solubilization of membrane proteins can be beneficial, as they have already led to the identification of unknown interaction partners [118], they can interfere with the biological activity of the protein. Often the results depend on the detergent used and its ability to denature the protein [91, 127]. Therefore, reconstitution should always be considered a second technique to confirm the results

initially obtained in detergents. Table 1 shows large differences between measurements in detergents and proteoliposomes for the transporter *Ec*MacAB [83, 91, 118]. During the last decades, researchers have developed alternative methods, including stabilization of the membrane proteins in bicelles or nanodiscs [128]. Bicelles are artificial lipid bilayers used as model membranes in biochemical and biophysical studies and are formed by the self-assembly of lipids in an aqueous solution. Nanodiscs are small lipid bilayers stabilized by scaffold proteins. These methods can help study these important proteins and should be further considered for *Pp*PvdRT-OpmQ [79, 128]. Another essential factor for a functional tripartite complex is the outer membrane protein. Unfortunately, we could not further analyze the *Pp*PvdRT system in complex with the OMP *Pp*OpmQ. However, a recent study on *Ec*MacAB was able to provide helpful information on the formation of a tripartite efflux system using reconstituted *Ec*MacB and *Ec*MacA in lipid nanodiscs and proteoliposomes containing the OMP *Ec*TolC simultaneously [119]. Such setups should be considered for further future investigation of the *Pp*PvdRT-OpmQ system.

5.3 Tripartite efflux systems and their contribution to pyoverdine secretion

In addition to the biochemical characterization of PvdRT-OpmQ, this work aimed to identify additional pyoverdine secretion systems. During this work, several systems were investigated to identify transporters involved in pyoverdine secretion. In addition to the *Pp*TtgABC and *Pp*ParXY systems (Chapter 3, Chapter 4), several other systems belonging to different transporter classes were tested by phenotypic characterization and growth analysis (Chapter 4, Figure 1). Figure 10 shows a predicted interaction network between all the systems studied for this work. Most of the proteins appear to be closely linked, with only a few outliers, such as the components of the T6SS. Since the T6SS represents a different class of membrane-integrated transport proteins capable of secreting effector proteins [129], it is not necessarily expected to interact with classical efflux pumps. Nevertheless, it is involved in pyoverdine secretion in other *Pseudomonas* strains [41], wherefore it was included in this analysis. Most of the systems studied belong to the RND transporter class. In addition to the T6S and RND systems, several ABC and MFS transporters and outer membrane porins were examined for their impact on pyoverdine secretion (Chapter 4). An initial screening for potential candidates resulted in several strains with difficulties growing under iron-depleted conditions. Some of the additional deletions in the genetic background of Δ*pvdRT-opmQ*Δ*mdtA* (PP_0803, PP_3302 and PP_5173, PP_2558, and the T6SS) showed minor difficulties in growing even in complex medium. It is important to consider that membrane-integrated proteins

affect the surrounding environment [130], and it is possible that the deletion of just one of these proteins can have significant effects on the cell integrity and therefore cause growth defects of respective mutants.

The interaction network (Figure 10) suggests a close functional relationship between almost all of these systems, which explains the difficulty in identifying a specific pyoverdine transport system. Although pyoverdine secretion could never be prevented entirely, the involvement of several systems was detected in *P. putida* KT2440. The inner membrane proteins of the systems most important for pyoverdine secretion, *Pp*PvdT and *Pp*MdtC (highlighted in bold), and the *Pp*ParXY system (PP _3456), are located in the center of the network.

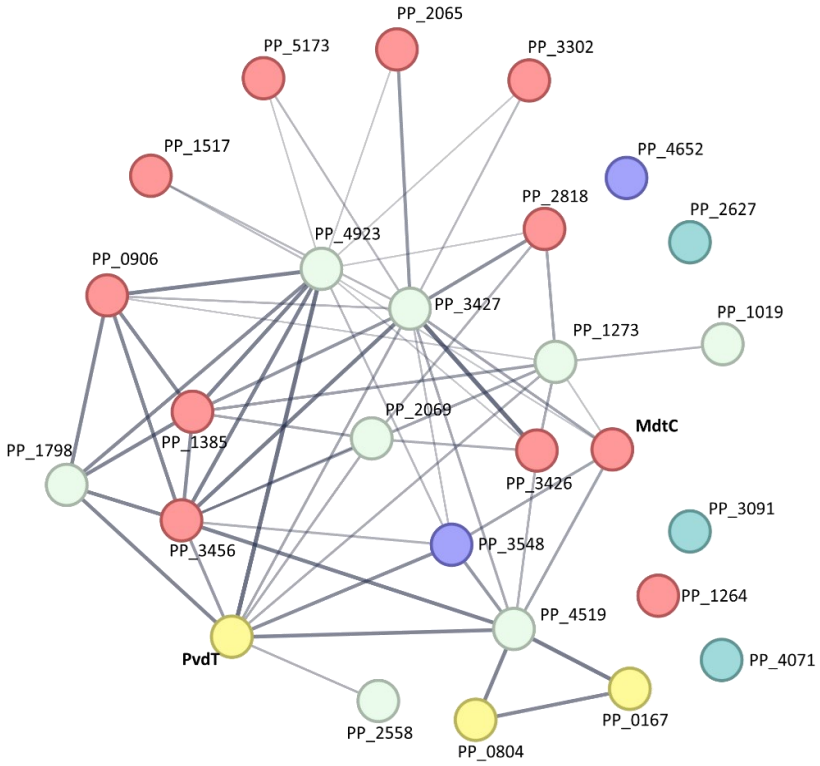


Figure 10: **Illustration of the interaction network of the different efflux systems studied in this work concerning pyoverdine secretion.** The tight interaction patterns are highlighted and color-coded to represent different groups of proteins: yellow, ABC transporters; light green, outer membrane proteins/porins; cyan, subunits of the T6SS system; red, RND transporters; purple, MFS transporters. The thickness of the lines indicates the level of support for each interaction as determined by data from the String database [122] and protein identifiers for each node (full STRING network, confidence edges, all active interaction sources, medium confidence). Even though the adapter proteins were deleted for several systems, the network was designed with the inner membrane proteins to allow for comparable prediction.

All three systems present multiple interaction partners, but it is still unclear if they can interact with other systems and how one system can restore the original function of a disrupted system. Further analysis using a bacterial two-hybrid assay [131] could

improve the general understanding of the interactions of these pumps. A good example is the OMP TolC of *E. coli*. This protein is encoded as a single gene in the genome. It plays a crucial role by interacting with several systems belonging to the ABC, RND, and MFS transporters, but with different levels of transport efficiency [132]. Since it has several interaction partners, *Ec*TolC and its associated systems must be tightly controlled in a complex mechanism. Transport systems are located all over the chromosome; therefore, studying the regulatory mechanisms of efflux pumps and their genetic environment is an essential topic for future research.

A neighboring regulator may not only control the associated operons by up or down-regulating them. They may have broader effects (direct and indirect) on the overall processes and expression of multiple efflux pumps. Specific examples include the second-site revertant strains identified for the Δ*ttgB* mutant discussed in Chapter 3 [102]. The *Pp*ParXY system also contains an upstream-located TCS, which has not been further investigated yet (Chapter 4).

5.4 TtgABC is a multidrug tripartite efflux pump whose substrate spectrum does not include pyoverdine

The TtgABC system, in particular, has been subject to investigation due to its relation to the Mex systems of *Pseudomonas* and its comprehensive substrate profile, which includes solvents and antibiotics [30, 97]. The induction of the MexAB operon of *P. aeruginosa* by iron was observed [104, 133], indicating a pivotal role of this RND efflux system in intracellular iron regulation. Therefore, it has been proposed as a siderophore transporter several times [104, 110, 133], but its involvement was later disproven for *P. aeruginosa* PAO1 [51]. Studies showed that strains of *P. aeruginosa* encode an OrfABC, later known as MexAB-OprK, which upon deletion, renders the strain susceptible to different classes of antibiotics, with poor growth under-iron depletion in 2,2'bipyridyl and minimal pyoverdine production. Additionally, the gene upstream of the operon *Pa*OrfABC shares homology with the Fur repressor of *E. coli* (explained in 1.4). Simultaneously, *Pa*OprK (later OprM [134]), the gene of the outer membrane protein of the system, proved to be overexpressed in a mutant with increased resistance towards 2,2'bipyridyl [104, 133]. The MexAB system of *P. aeruginosa* shares a protein identity of 67 and 78% of TtgAB of *P. putida*, respectively [123]. It has been proposed that the MexAB-OprM system of *P. aeruginosa* may assist in acquiring nickel and zinc through the transport of the metallophore pseudopaline, although it has been proven that it does not transport siderophores. *Pa*MexAB is therefore thought to play a role in ion acquisition in

pseudomonads [135] and potentially in the secretion of siderophores by related strains. Due to the homology and indications given by the MexAB system of *P. aeruginosa*, the *Pp*TtgABC system was selected as the first system after the identification of PvdRT-OpmQ and MdtABC-OpmB [90] for further analysis. Initial attempts to demonstrate active pyoverdine transport by TtgABC were misleading for two reasons: first, pyoverdine production was indeed reduced under iron limitation, leading to decreased siderophore secretion (Chapter 3). Rendered siderophore secretion was caused by the downregulation of pyoverdine-related genes (*pfrI*, *pvdL*, and *fpvA*) (Chapter 3, supplementary data). Secondly, under physiological stress conditions, such as the selection process used to generate deletion mutants applied in several studies [85, 90, 102], *P. putida* strains can stop pyoverdine production completely. This non-fluorescent phenotype is so far not further investigated and needs to be characterized. To further analyze the phenotype, Δ*ttgB* strain was compared the control strain 3E2, which does not produce pyoverdine. Additionally, the Δ*ttgB* deletion was introduced in the control strain 3E2 (not shown in Chapter 3). Both experiments showed that the deletion of *ttgB* causes a phenotype under iron-limitation, which is related to 2'2-bipyridyl and not pyoverdine or oxidative stress via the Fenton reaction. The latter effect was tested by analyzing the expression of genes involved in oxidative stress response, *sodA*, *sodB* and *ohrR*, and the ferric uptake regulator Fur (Chapter 1.4). Additionally, the corresponding phenotype could be complemented by a pUCP-*ttgB* plasmid introduced in to the *ttgB* deletion strain. These data indicated, that a malfunction of the RND transporter causes drastic phenotypes in response to the iron chelator, which can be also concluded from the fact, that *ttgB* gene is upregulated as a response to 2'2-bipyridyl.

To confirm the phenotype caused by a deletion of *ttgB*, the effect of Phe-Arg-β-naphthylamide (PAβN) in combination with 2,2'-bipyridyl was analyzed, indicating an efflux-mediated resistance to this specific iron chelator. PAβN is a cationic agent that incorporates into the LPS layer of the outer membrane and might impact the integrity of the OM [136]. At the same time, it is a substrate for RND efflux pumps [137]. The severe effects on growth behavior and the resulting phenotype could be attributed to the influence of 2'2-bipyridyl on cell metabolism and subsequent efflux by the *Pp*TtgABC system, which was disrupted. The observed phenotype is consistent with the increased susceptibility to 2'2-bipyridyl upon deleting MexAB-OprM in *P. aeruginosa* [104]. The accumulation of the iron chelator in the cell inhibited intracellular processes by removing ions such as copper and iron from important enzymes. As a result, several other processes that rely on these ions as cofactors, such as proline synthesis, were downregulated, and intracellular ATP levels decreased measurably (Chapter 3).

This observation raises the question of whether it is still appropriate to use potentially toxic iron chelators to create iron-deficient conditions for research. The use of the easily available and cheap iron chelator 2'2-bipyridyl instead of human apo-transferrin [138], as it is done in laboratories around the world, is a perfect example of the importance of understanding the roles of efflux pumps: the use of toxic 2'2-bipyridyl can subsequently lead to misinterpretation of the results obtained in iron deficiency and simultaneous absence of the *Pa*MexAB and *Pp*TtgABC system or its homologs since the interference of 2'2-bipyridyl on metabolic and other internal processes inside the cell cannot be excluded. As mentioned above, the involvement of MexAB in pyoverdine secretion in *P. aeruginosa* was hypothesized in 1993 when experiments were performed with Bip [104]. More than ten years later, this hypothesis was denied, and it was proven that this system is not involved in pyoverdine secretion. Later, mutants of the *Pa*MexAB system were analyzed in DCAA medium, a metal-free version of the CAA medium used in Chapter 4. Here, the deletion of *Pa*MexAB did not result in significantly decreased siderophore secretion [51]. It is possible that the phenotypes observed in *Pa*MexAB mutants in this study [133] were due to the absence of a detoxification system rather than the absence of pyoverdine transporters-which could have been avoided by changing the media conditions instead of using Bip. Another important observation was that if the TtgABC system was deleted in *P. putida* KT2440, revertants emerged that could grow on a medium supplemented with 2,2'-bipyridyl. This new phenotype was later associated with a mutation in the gene PP _2827 that resulted in increased expression of PpMexEF-OprN, controlled by PP_2826 [102]. A similar effect has already been discussed for *Pa*MexAB-OprK [133]. This interaction between the two genes is an excellent example of the regulatory cascades underlying efflux systems, as described in 1.4. It shows how quickly microorganisms can adapt to environmental changes and respond to selective pressures.

Although TtgABC has not been shown to secrete pyoverdine, this system is capable of transporting a wide range of substrates, including deoxycholic acid, 2,2'-bipyridyl, and its natural derivative caerulomycin A produced by *Streptomyces caeruleus*. The data presented in Chapter 3 underscore the natural role of this efflux system, which, like presumably most efflux systems in Gram-negative bacteria, evolved many millennia before antibiotics were first used. Further studies are needed to determine whether other natural bipyridyl derivatives with antibiotic activity, such as other caerulomycins and cyanogrisides [139], are transported by *Pp*TtgABC.

5.5 TtgABCs ancestral role: natural defender against 2,2’ bipyridyl derivates?

Interestingly, and in contrast, structurally related substances such as biphenyl (which lacks only the two amines) can be degraded by *P. putida* B6-2 to benzoic acid and subsequently used for the TCA cycle [29]. Another interesting observation is that the pseudomonads *P. fluorescens* and *P. indigofera* produce intracellular insoluble blue pigments, lemonnierin, and indigoidine. Both compounds are derivatives of 3’3-bipyridyl. Even though these pigments are associated with the cells due to their insolubility [3], a possible native function of *Pp*TtgABC in the transport of derivatives of these pigments cannot be excluded. These examples show, on the one hand, how accurate the discrimination of a single transporter towards different substrates is and, on the other hand, how sensitive the equilibrium in a bacterial cell can be and how closely the expression of an efflux system is related to internal metabolic processes.

As described above, the substrate spectrum of RND pumps includes antibiotics and various other substances, indicating an essential role of these systems for microbe-microbe interactions in nature [140]. Efflux pumps can be dated back to times before the development of antibiotics [86], and studies have shown that duplication events of PACE [80] and RND transporters occurred early in the evolutionary history of bacteria. The $MdtB_2C_1$ heterotrimer evolved during the separation of β- and γ-proteobacteria at least 1.6 billion years ago, raising the question of what were the original substrates of these efflux systems and why did such duplication events provide an evolutionary advantage for bacteria [95]? Natural substrates are most likely host- and microbe-derived substances in the microbes' natural habitat. Bile acids, for example, are found in the human gut and are transported by the AcrAB system of *E. coli* and other bacteria. Inactivation of this pump in the gut microbe renders the strain hypersensitive to intestinal acids [72, 78].

The study of the *Pp*TtgABC system supports this hypothesis since, on the one hand, it is present in the strain *P. putida*, which colonizes a variety of plant tissues and interacts with them and other organisms [141]. On the other hand, the system is responsible for the detoxification of deoxycholate and 2'2-bipyridyl and its natural derivatives (Chapter 3, Figure 11). Deoxycholate is present in the human intestine. It is metabolized from cholic acid by intestinal bacteria in the gut [142]. At the same time, it is structurally similar to mammalian steroid hormones. Evidence exists for efflux systems that actively transport human steroid hormones in *E. coli* [143]; therefore, this system may take over a comparable role in *P. putida* KT2440.

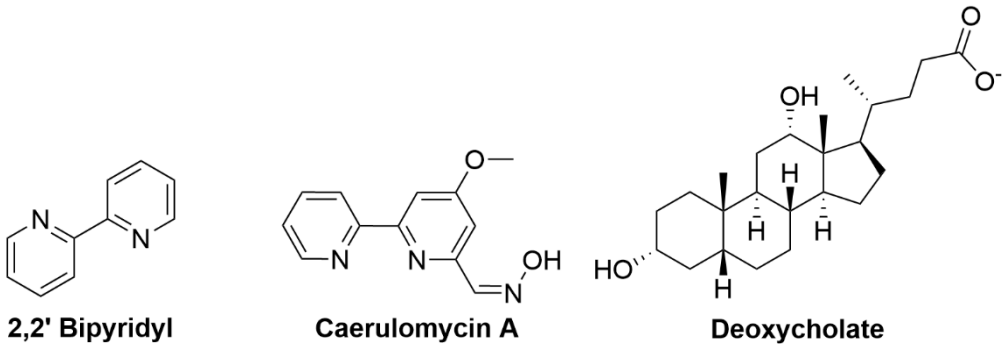


Figure 11: **Chemical structures of the substrates of the TtgABC system of *P. putida* KT2440.** 2,2' bipyridyl is an iron chelator commonly used in laboratories, while caerulomycin A is a natural derivative produced by *Streptomyces caeruleus* [85]. Deoxycholate is a cholic acid degradation product metabolized by intestinal bacteria [144]. Structures PubChem IDs: 1474,159917, 222528. Structures were modified using ChemDraw Version 19.1.1.21.

Additionally, it is imaginable that the substrate spectra of these systems evolve continuously. For example, the iron chelator presented in Chapter 3 is used to produce diquat, a toxic herbicide. Even though it is toxic, this herbicide has no visible effect on soil microbial fitness when only applied once [145], indicating that the bacterial community has a defense mechanism against this herbicide. Whether this is related to the fact that a particular population of the soil community is indeed resistant to diquat by enabling transport through a tripartite efflux system, such as the TtgABC system or homologs, seems possible. At the same time, it is likely that the compound is degraded and thereby effectively removed from the population. Introducing diquat and other new herbicides into agriculture might represent the selective pressure that enables efflux systems such as the TtgABC system to expand their substrate spectra towards these chemicals. Therefore, the physiological role of the TtgABC system and its homologs in pseudomonads and related species is essential for studying iron metabolism, iron depletion, synthesis, and secretion of siderophores. The lack of only one efflux system involved in the detoxification of bipyridyl derivatives can have drastic effects on bacterial metabolism (Chapter 3), highlighting the importance and necessity of efflux pump inhibitors (EPIs) for future therapeutic strategies [146, 147]. In many cases, it is unnecessary to inactivate all efflux pumps simultaneously, but only one main efflux system with a known target to convert non-functioning antibiotics back to bactericidal ones.

5.6 ParXY as a putative siderophore transport system important under iron limitation

Recently, the ParXY system in *P. putida* KT2440 and its resistant isolate HPG-5 was described as involved in multi-resistance to several unrelated antibiotics and dyes. The closest homolog of this system in *P. aeruginosa* is the MexCD-OprJ system (> 50% identity) [16], while in *E. coli*, the closest homolog is the AcrAB system (48% identity) (Chapter 4, supplementary data). The latter system has been identified as one of the major players in enterobactin secretion in *E. coli* [100]. Of the 12 efflux pumps identified in *P. aeruginosa*, five belong to the group of Mex systems [73], all of which have orthologous systems in *P. putida* KT2440 [21]. As discussed in Chapter 1.2, iron availability is crucial for host colonization, so it is tempting to speculate on the involvement of at least one of the different Mex systems in the transport of pyoverdine.

To rule out a possible role of the products of PP_3455/56 (ParXY), deletion mutants were generated [90]. A strain lacking all three systems (Δ*pvdRT-opmQ* Δ*mdtA* Δ*parX*) secreted significantly less siderophore than the double deletion strain [148]. At the same time, reduced production and increased accumulation of the siderophore were observed. The system tends to depend on iron availability, even though it lacks a binding site for PvdS (PfrI) [148]. Taken together, these data from Chapter 4 strongly suggest an active involvement of the ParXY system in the secretion of the siderophore under the condition of iron limitation and a lack of the main systems PvdRT-OpmQ and MdtABC-OpmB.

Nevertheless, the effect is not as drastic as for the previously identified *Pp*PvdRT-OpmQ and *Pp*MdtABC-OpmB systems, and secretion has not yet been completely abolished. Whether deletion of ParXY stimulates feedback mechanisms such as up-and down-regulation of other secretion systems resulting in reduced pyoverdine secretion requires further investigation. The experiments were performed in a casamino acid medium (CAA), representing substantial iron limitation but not total iron depletion [148]. The induction of intracellular oxidative stress-related processes by the Fenton reaction was not further investigated because such reactions are not expected. The high affinity of pyoverdine for iron should abolish excessive iron within the cell.

Nevertheless, the accumulation of siderophores may have pleiotropic effects by, among other things, chelating iron from enzymes of the electron transport chain [149]. This effect could indeed cause a similar effect as the Δ*ttgB* phenotype in Chapter 3. By adding external pyoverdine as a control, the phenotype caused by a triple deletion of Δ*pm*Δ*parX* could be rescued [148]; therefore, intracellular toxicity of the siderophore seems unlikely. Additionally, the hypothesis of a possible iron deficiency is further confirmed by the

observation that the addition of 1 µM external $FeCl_3$ rescues the phenotype triggered in CAA medium to a similar extend, than external pyoverdine. Taken together, these results are the basis of proposing the ParXY systems as a new putative pyoverdine transport system [148]. What lastly stands out is the fact, that the ParXY system has an unusual genetic organization (Figure 8): the inner and membrane fusion protein are allocated in an operon with an upstream encoded TCS. This characteristic is not shared with the two other systems *Pp*PvdRT-OpmQ and *Pp*MdtABC-OpmB.

5.7 The ParXY system lacks an outer membrane protein but encodes an upstream two-component system

The investigation of putative outer membrane proteins was not included in Chapter 4. The information, whether one of the proposed outer membrane proteins (PP_3427, PP_4923, PP_4519, PP_1798, predicted by string-db.org [122]) or even the proposed TtgC [16] acts as a porin for the tripartite efflux system is not available yet. The physical interaction of the *Pp*ParXY system with the *Pp*TtgC outer membrane porin needs to be evaluated further (e.g. by a BACTH [131]). This information would be helpful in understanding the abilities of efflux systems to transport variable substrates. It would be also interesting to evaluate, whether the system has a similar substrate spectrum as the *Pp*TtgABC (Chapter 3), in case *Pp*TtgC is an interaction partner of *Pp*ParXY.

PP_3454 encodes a response regulator (RR), and PP_3453 is a histidine kinase (HK). Due to its low level of conservation compared to other TCSs, such as ParRS from *P. aeruginosa* PAO1, the system was later named PpeRS (*P. putida* efflux regulator/ sensor), with a yet undetermined function in regulating efflux systems. For the phenotype of a Δ*ttgB* deletion mutant in Chapter 3, a complementing effect by the MexEF-OprN system was shown upon mutation of one of the regulatory proteins (PP_2827) [102]. At the same time, five TCSs were found to actively regulate the expression of RND efflux systems in *P. aeruginosa* [73]. The TCS upstream of PP_3455/56 is predicted to interact with other TCSs in the strain (according to string-db.org [122]) and thus may play a role in the up-or-down-regulation of other systems upon deletion of the ParXY system. Recently, in a resistant isolate of *P. putida*, it was shown that deletion of the PP_3453/54 genes resulted in repression of *ttgA* and *parX*, whereas repression of PP_2817 (*mexC*) was abolished [16]. Two-component systems have been shown to play crucial roles in regulating RND systems, such as the CzcCBA of *P. putida* [72]. It stands out that deletion of the gene PP_3455 encoding the adapter protein of the ParXY system triggers a phenotype with an increased resistance towards chloramphenicol (Chapter 4,

supplementary data). A similar effect has already been shown [16]. Inactivation of one RND system usually is believed to result in decreased resistance towards possible substrates of the respective pump. The opposite effect strongly indicates the involvement of another efflux system, which in consequence, had to be activated or upregulated by an unknown regulatory mechanism, e.g., a TCS. Whether the deletion of *parX* subsequently leads to the upregulation of another system by PpeRS needs to be further evaluated. Chloramphenicol is indeed one substrate of the TtgABC system of *P. putida* [16, 97, 98], and regulation by PpeRS has been shown [16]. Still, an involvement of TtgABC in pyoverdine secretion has been excluded in Chapter 3, wherefore the reduced secretion of the triple deletion strain is finally attributed to the deletion of *parX* [148].

The possibility of upregulation of another unidentified system, originally responsible for pyoverdine secretion, upon deletion of *parX* remains possible. Further investigation of genes interacting with the gene PP_3453 using the String Database [122] revealed two transport proteins, that are predicted to interact with ParX: PP_4453 and PP_4492. Both proteins are hardly characterized, nor is there any evidence of a relation to pyoverdine secretion. Therefore, further future research in this area could focus on the role of those two transporters.

5.8 Final conclusion

Results presented in the previous chapters discussed the complex roles and interactions of tripartite efflux pumps in *P. putida* KT2440. First and initial analysis of the biochemical interaction could be shown for both the interaction between *Pp*PvdT and *Pp*PvdR and the complex with its ligand pyoverdine, supporting the idea of *Pp*PvdRT-OpmQ as a main efflux system for this siderophore by initial biochemical data (Chapter 2, Figure 12, green box).

Besides this system, other systems were subject to investigation: the *Pp*TtgABC system (Chapter 3) does not contribute to pyoverdine secretion, but its substrate spectrum could be further extended by identifying several (toxic) substrates of this system (Figure 12, rose box), such as 2’2-bipyridyl, which in consequence of a deletion of *Pp*TtgABC accumulated inside the cells and caused a collapse of the intracellular balance. This data raised the question of the costs and benefits of using such iorn chelators.

Lastly, the *Pp*ParXY system was proposed as a new pyoverdine transport system which seems to work closely together with the two other described systems, *Pp*PvdRT-OpmQ and *Pp*MdtABC-OpmB (Chapter 4, Figure 12, blue box and right). However, these results also represent only a part of the complex underlying transport mechanisms that *P. putida*

possesses. Therefore, much more research is needed in the future on the one hand, to understand the physiological role of efflux pumps better and, on the other hand, to develop new strategies against pathogenic bacteria.

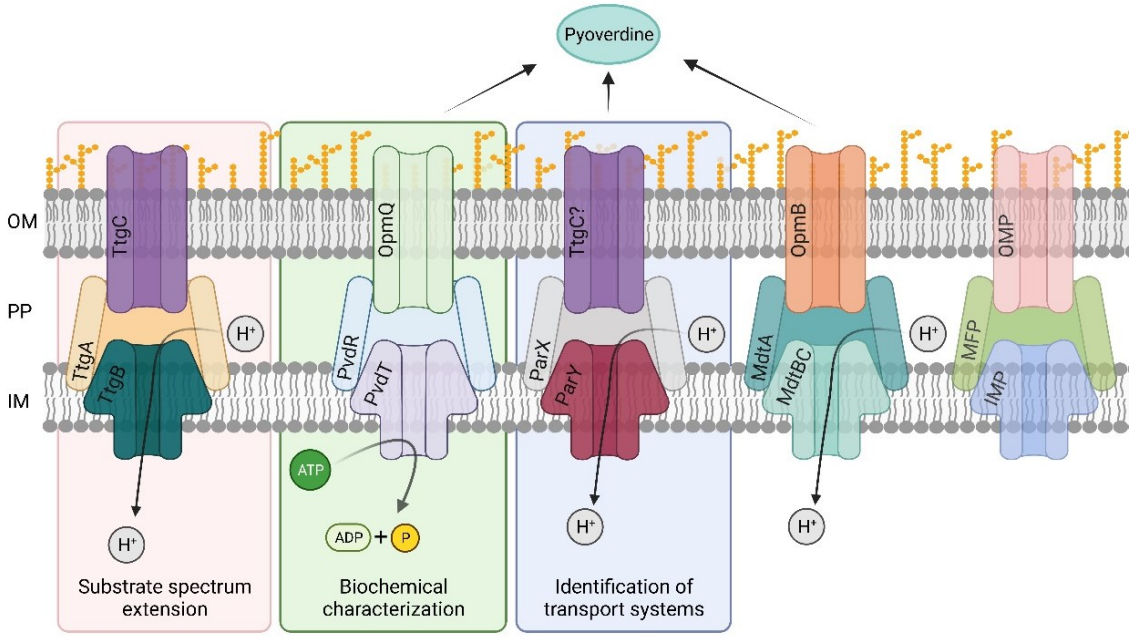


Figure 12: **Concluding overview and summary of systems investigated in this thesis.** Upon iron limitation, *P. putida* KT2440 secretes the fluorescent siderophore pyoverdine into its environment (cyan molecule) to chelate iron. The transporter classes of ABC and RND are known to be involved in this process. The two transport systems PvdRT-OpmQ and MdtABC-OpmB have been described for the transport of pyoverdine in *P. putida* KT2440 [90]. This thesis aimed to characterize (green box) and identify tripartite efflux systems involved in pyoverdine secretion and secretion of different substrates (blue and rose box). The interaction and kinetic behavior of the PvdRT-OpmQ system with its proposed ligand pyoverdine could be shown for the first time (green box, Chapter 2). Initial analysis of the main efflux system TtgABC could provide new data on the substrate spectrum of this pump (orange box, Chapter 3). Later, the ParXY system was identified to be involved in pyoverdine secretion if PvdRT-OpmQ and MdtABC-OpmB were missing (Chapter 4). The results from this thesis indicate that at least one more system is involved in the process of pyoverdine secretion (right). The figure was generated using BioRender.com.

6. Outlook

"The effect of efflux pumps needs to be considered in the design of future antibiotics and the role of inhibitors assessed in order to maximize the efficacy of current and future antibiotics." (Webber *et al.* 2003, [77])

This work provides new insights into the functioning of the ABC transporter *Pp*PvdRT-OpmQ and the two RND transporters *Pp*TtgABC and *Pp*ParXY, including enzyme kinetics, substrate binding, and substrate spectra. These results are consistent with previous research but also offer new perspectives on the origin of these pumps and the identification of potential new substrates. However, our understanding of the complex mechanisms and networks behind these efflux systems still needs to be completed, and further research is needed to develop effective strategies to combat pathogens. These results show that efflux systems may be genetically related but have distinct physiological functions in *P. putida* KT2440. Antimicrobial resistance is a major problem that is expected to cause 10 million deaths per year by 2050, underscoring the importance of finding new approaches to combat pathogens. Particular attention should be paid to strains with an already extended resistance spectrum, the so-called group of ESKAPE pathogens: ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****. aeruginosa*, ***E****nterobacter spp*., and ***E****. coli* [150]. Although efflux pumps were identified decades ago and MICs are available for many antibiotics, inhibitors of efflux pumps are still lacking [112], opening new opportunities to suppress parts of multidrug efflux systems and to combine this approach with antibiotic treatment. For example, sideromycins such as cefiderocol (Fetroja®) can actively import antimicrobial agents via siderophore absorption [151]. In combination with protease-cleavable linkers with strain-specific recognition, this strategy could be extended to various pathogens in the future [150]. However, the exact mechanism of action of sideromycins remains unclear, and they should only be used to target specific strains [44]. Other potential solutions to this problem include drugs that induce the formation of cheaters in bacterial populations, thereby reducing overall fitness [5], new advanced efflux pump inhibitors [77], and lastly, more complex techniques such as RNA interference and microRNA mimetics to downregulate the expression of efflux systems [87]. Successful attempts have been made using CRISPR-Cas to inactivate efflux pumps [152] or β-lactamases [153], representing another promising strategy.

Biochemical characterization of efflux systems of especially pathogenic bacteria needs to be improved more by designing new and improved detergents and techniques, such

as amphipols, that are protein specific and enable much easier purification processes than those used today. One recent example is the development of modified amphipols, which could be used to specifically purify the RND transporter AcrB of *E. coli* from membranes to perform cryoEM directly [154]. Such techniques harbor the potential to simplify the purification process significantly. Reconstitution of *Pp*PvdRT-OpmQ followed by transport assays with all three pump components could confirm the results gained in this thesis. Later on, the resolution of the crystal structure of PvdRT-OpmQ, ideally with bound pyoverdine, would further widen our knowledge of tripartite efflux pumps and their substrates.

Furthermore, the question of which additional pump is responsible for the secretion of the siderophore remains unanswered, leading to speculation that several systems could simultaneously take over the function of the missing pyoverdine transport system. To further investigate pyoverdine secretion, simultaneous deletions of the three systems, *Pp*PvdRT-OpmQ, *Pp*MdtABC-OpmB, and *Pp*ParXY, should be considered along with new and unstudied pumps. The pyoverdine non-producer 3E2 as a control strain shows that *P. putida* KT2440 is viable without functional pyoverdine secretion under iron-replete conditions. However, there is still no indication whether a strain in which multiple pumps have been simultaneously deleted is still physiologically viable.

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Supplemental information Chapters 1, 5 and 6

The cover image (LMU siegel) was downloaded from the university website using the following link for the LMU *Serviceportal*:

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