

Ludwig-Maximilians-Universität München



**Pyoverdine production in the pathogen  
*Pseudomonas aeruginosa*: a study on  
cooperative interactions among individuals  
and its role for virulence**

**Dissertation**

der Fakultät für Biologie  
der Ludwig-Maximilians-Universität  
München **vorgelegt von**

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**April 2017**

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**Tag der Abgabe:** 03.04.2017

**Tag der mündlichen Prüfung:** 09.06.2017

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## Preface

### 1.1 Eidesstattliche Erklärung/Statutory Declaration

#### Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig 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 vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

#### Statutory Declaration

Herewith I affirm that the presented dissertation was made autonomously without illegal help. Moreover, I certify that I have not previously submitted a dissertation without success and that a doctoral examination was not done in the past without success. The presented dissertation was not submitted completely or essential parts of it to another examination board.

München, den: 17.07.2017

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Michael Weigert

## **1.2 Publications and Manuscripts Originating**

### **From this Thesis**

#### **Chapter 3**

Ross-Gillespie, A.\*, Weigert\*, M., Brown S.P, and Kümmerli R., 2014, Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment, *Evolution, Medicine, and Public Health* (2): 18-29.

\*these authors contributed equally to this work

#### **Chapter 4**

Weigert, M., Ross-Gillespie A., Leinweber A., Pessi G., Brown S.P., and Kümmerli R., 2017, Manipulating virulence factor availability can have complex consequences for infections, *Evolutionary Applications* 10 (1): 91-101.

#### **Chapter 5**

The physical boundaries of public goods cooperation between surface-attached bacterial cells, 2017, *Proc. R. Soc. B* 2017 284 20170631

### **Publications and Manuscripts not Included in this Thesis**

Schiessl, K., Ross-Gillespie, A., Cornforth, D.M., Weigert M., Bigosch C., Brown S.P., Ackermann M., Kümmerli R., 2016, Individual- versus group-optimality in the production of secreted bacterial compounds, submitted to *Evolution* (in revision).

## **1.3 Contributions to Publications and Manuscripts Presented in this Thesis**

### **Gallium-Mediated Siderophore Quenching as an Evolutionarily Robust Antibacterial Treatment**

Michael Weigert and Rolf Kümmerli designed the experiments. Michael Weigert conducted all *in-vivo* experiments. Adin Ross-Gillespie and Michael Weigert conducted *in-vitro* experiments together. Adin Ross-Gillespie, Michael Weigert and Rolf Kümmerli analysed the data. Adin Ross-Gillespie, Rolf Kümmerli, Michael Weigert and Sam Brown wrote the paper.

### **Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections**

Michael Weigert, Ross-Gillespie and Rolf Kümmerli designed the experiments. Michael Weigert conducted all experiments except for figure 1, which was conducted by Anne. Leinweber Anne Leinweber also provided input for primer design and help to conduct the qPCR experiments. Gabriella Pessi provided the protocol for RNA extraction and helped to analysed qPCR data. Michael Weigert and Adin Ross-Gillespie analysed the data. Michael Weigert, Adin Ross-Gillespie, Sam Brown, Gabriella Pessi, Anne Leinweber and Rolf Kümmerli wrote the paper.

# **The Physical Boundaries of Public Goods Cooperation Between Surface-Attached Bacterial Cells**

Michael Weigert and Rolf Kümmerli designed the experiments. Michael Weigert conducted the experiments and wrote the code for batch analysis of microscopy pictures. Michael Weigert analysed the data. Michael Weigert and Rolf Kümmerli wrote the paper.

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Michael Weigert

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Prof. Rolf Kümmerli

## 1.4 Abbreviations

AHL	N-Acyl homoserine lactones
CF	Cystic fibrosis
ETA	ExotoxinA
FUR	Ferric uptake regulator
GFP	Green fluorescent protein
HS	Human serum
$K_s$	Stability constant (strength of complex formation)
$K_f$	Dissociation constant (affinity between a ligand and a protein)
mcherry	Red fluorescent protein
PCR	Polymerase chain reaction
pvd	Pyoverdine
QQ	Quorum quenching
qPCR	Quantitative real-time PCR
QS	Quorum sensing

## 1.5 Summary

Opportunistic pathogens like *Pseudomonas aeruginosa* become increasingly resistant to antibiotics, and therefore represent a major threat to patients. Thus, we urgently need new approaches to fight multi-resistant pathogens. It has been suggested that, instead of targeting vital cell mechanisms, virulence factors could be inhibited with so-called anti-virulence treatments. These treatments are believed to impose lower selection pressure on the pathogen and would thereby reduce the risk of resistance development. In this thesis, we aim to extend the anti-virulence approach by targeting a secreted virulence factor that is cooperatively shared between bacteria. For many pathogens, cooperation is essential to infect hosts, and is often mediated by secreted, publically shared virulence factors. Cooperating individuals can be exploited by individuals, which do not contribute to cooperation, but reap the benefits from it, so-called cheaters. By targeting a cooperatively shared virulence factor, the cooperating community becomes phenotypic cheaters and every individual, that resumes cooperation (e.g. by developing resistance), will be exploited immediately and thus resistance is not favoured by natural selection. Such a treatment can become evolution proof.

We tested this idea by inhibiting the cooperatively shared virulence factor pyoverdine. Pyoverdine is the main siderophore of *P. aeruginosa*, which is deployed in severely iron-limited environments to assure sufficient supply of this essential nutrient. Pyoverdine facilitates pathogenic growth at the infection site. In chapter (3) we experimentally tested a promising candidate, the transition metal gallium, as an evolution proof anti-virulence treatment, that targets pyoverdine. Gallium effectively curbed the virulence of *P. aeruginosa* in an insect model. Moreover, while antibiotics lost their efficacy rapidly in an evolution experiment, *P. aeruginosa* did not show signs of resistance to gallium.

Next, we tested if and how such interference with virulence factor availability (pyoverdine) feeds back on the pathogen, its regulatory network and the host (chapter 4). We found complex relationships between these variables. While the link between virulence factor availability and virulence was positive, pyoverdine availability did not correlate monotonously with pathogen growth within the host. The amount of available virulence factor influenced the expression of virulence factors, that are regulatorily linked. Additionally, it triggered differential host immune responses. These findings highlight the necessity to closely evaluate the effects of any anti-virulence drug on the pathogen and the host, in order to design effective drugs with a predictive treatment outcome.

The concept of evolution proof anti-virulence treatments builds (among others) on the assumption that the targeted virulence factor is collectively shared between individuals. Although pyoverdine cooperation has been extensively studied in the last decade, almost all studies feature experiments in batch cultures. However, little is known about whether the insights from batch culture experiments can be transferred to infections. In the host, cell numbers might be lower and bacteria might interact on the micrometre-scale in a spatially structured environment where diffusion of a shared virulence factor, and thus shareability, could be limited. Therefore, we investigated pyoverdine sharing between individuals, attached to a surface, at the level of single cells by using fluorescent microscopy, and experimentally tested the physical boundaries of pyoverdine sharing (chapter 5). We found that even in highly viscous environments, pyoverdine is publically shared over a considerable distance. These findings validate the assumption that pyoverdine is cooperatively shared, even in viscous environments, such as experienced in infections, and therefore indicates that anti-virulence treatments targeting pyoverdine (e.g. via gallium), could indeed be evolution proof.



## 1.6 Zusammenfassung

Opportunistische Erreger wie *Pseudomonas aeruginosa* entwickeln stetig Resistenzen gegen eine Vielzahl von Antibiotika und bedrohen immer öfter die Gesundheit von Patienten. Deshalb bedarf es dringend neuer Ansätze, mit denen wir solche multi-resistenten Erreger bekämpfen können. Eine Möglichkeit wäre, anstatt essentielle Zellmechanismen anzugreifen, Virulenzfaktoren direkt mit sogenannten Anti-Virulenzmitteln zu inhibieren. Man geht davon aus, dass solche Mittel einen geringeren Selektionsdruck auf Bakterien ausüben und deswegen weniger Resistenzen hervorrufen sollten. In dieser Arbeit wollen wir diesen Ansatz weiter entwickeln und schlagen vor Virulenzfaktoren zu inhibieren, welche sekretiert und zwischen Individuen kooperativ geteilt werden. Wenn Erreger einen Wirt infizieren wollen, müssen viele von ihnen kooperieren, was oft durch die Sekretion von kollektiv geteilten Virulenzfaktoren geschieht. Kooperation kann jedoch von Individuen ausgenutzt werden, welche die Vorteile von kooperativem Verhalten genießen, ohne sich aber daran zu beteiligen, sogenannte "Cheaters". Indem ein kooperativ geteilter Virulenzfaktor inhibiert wird, verwandelt sich die Kolonie in phänotypische "Cheaters", welche jeden ausnutzen werden, der wieder zu kooperieren beginnt (indem er z.B. resistent wird) und folglich sollte Resistenz gegenselektioniert werden. Solch ein Mittel könnte daher in der Tat evolutionär robust sein.

Diesen Ansatz haben wir anhand des kooperativ geteilten Virulenzfaktors Pyoverdine untersucht. Pyoverdine ist das primäre Siderophor von *P. aeruginosa*, welches unter starker Eisenlimitierung sekretiert wird, um eine ausreichende Versorgung mit diesem essentiellen Nährstoff sicher zu stellen. So trägt es beispielsweise zum Wachstum von *P. aeruginosa* in Wirten bei. In Kapitel 3 haben wir einen vielversprechenden Kandidaten für ein evolutionär robustes Anti-Virulenzmittel getestet. Das Übergangsmetall Gallium Pyoverdine und

reduzierte in unseren Versuchen mit Insekten die Virulenz von *P. aeruginosa* in der Tat sehr effektiv. Außerdem ist es nach einem Evolutionsexperiment weiterhin gegen *P. aeruginosa* wirksam, wohingegen Resistenzen gegen konventionelle Antibiotika schnell aufkommen.

Als nächstes haben wir untersucht, wie sich eine solche Manipulation der Verfügbarkeit des Virulenzfaktors, auf den Erreger, sein genetisches regulatorisches Netzwerk und den Wirt auswirkt (Kapitel 4). Wir haben komplexe Beziehungen zwischen diesen Variablen festgestellt. Während die Verbindung zwischen Virulenzfaktor-Verfügbarkeit und Virulenz positiv war, korrelierte die Pyoverdin-Verfügbarkeit nicht monoton mit dem Wachstum des Erregers innerhalb des Wirts. Zusätzlich beeinflusste die Virulenzfaktorverfügbarkeit andere Virulenzfaktoren, die mit Pyoverdin regulatorisch verbunden sind, sowie den Wirt. Diese Ergebnisse zeigen die Notwendigkeit auf, die Konsequenzen von Anti-Virulenzmitteln genau zu untersuchen, um einen wirksamen Ansatz zu finden, dessen Effekte sich gut vorhersagen lassen.

Das Konzept von evolutionär robusten Anti-Virulenzmitteln beruht unter anderem auf der Annahme, dass der inhibierte Virulenzfaktor Pyoverdine, kollektiv geteilt wird. Zwar wurde Kooperation mit Pyoverdine im letzten Jahrzehnt detailliert untersucht, aber fast alle Studien beinhalten Experimente auf Populationsebene. Allerdings weiß man bisher wenig darüber, inwieweit die Ergebnisse solcher Experimente sich auf Infektionen übertragen lassen. Dort kann die Zellzahl sehr gering sein und Bakterien kooperieren im Bereich von einigen Mikrometern. Unter diesen Bedingungen ist möglicherweise die Diffusion des geteilten Guts stark limitiert. Deswegen haben wir mithilfe eines Fluoreszenzmikroskops die Pyoverdine-Kooperation auf Einzelzellebene untersucht und deren physikalischen Grenzen experimentell ausgelotet (Kapitel 5). Wir fanden heraus, dass Pyoverdine unter viskosen Bedingungen über eine größere

Distanz geteilt wird. Diese Ergebnisse validieren die Annahme, dass Pyoverdine auch unter viskosen Bedingungen geteilt wird, und dass Anti-Virulenzmittel, die Pyoverdine inhibieren (wie z.B. Gallium), tatsächlich evolutionär robust sein könnten.

## CHAPTER 2

### Introduction

*It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.*

Alexander Flemming (1945)

## 2.1 Cooperation in Bacteria

Bacteria are highly social organisms, which deploy cooperative behaviours to overcome nutrient starvation, reproduce or infect a host. For instance, bacteria cooperate when they form biofilms, control community-wide phenotypes via quorum sensing, or when they secrete shareable compounds such as proteases and siderophores [1–5].

In biofilms, bacteria live together in a matrix of secreted extracellular polymeric substances to protect themselves from external influences, such as antibiotics [3, 6–8]. Biofilms are mainly composed of extracellular DNA, polypeptides and exopolysaccharides [9]. Biofilm-formation is thought to require a high degree of cooperation, since all members of the community engage in the costly secretion of these extracellular polymeric substances [3]. The protective effect of a biofilm (e.g. protection from antibiotic treatment [8]) is, however, beneficial for every member of the community and therefore, biofilm formation is seen as a cooperative trait [10].

Two of the most studied cooperative behaviours in bacteria are quorum sensing (QS), a communication tool for bacteria to regulate community wide phenotypes [11–18], and the production and secretion of siderophores [4, 19–24]. Siderophores are secreted, secondary metabolites with a very high affinity for iron, an essential but simultaneously severely limited nutrient in most environments, including hosts [25]. Since these traits (QS and siderophore secretion) are of particular relevance for this thesis, they will be discussed separately in section 2.2 and 2.3.

Bacterial cooperation can have a severe impact on the health of patients, since it often gives pathogens the necessary means to infect and spread within a host and to endure antibiotic treatment (e.g. biofilms) [17, 26–29]. For instance, 65–80% of all bacterial infections in humans are linked to biofilms and are therefore associated with high rates of antibiotic resistance [30–32]. In infections *Vibrio*

*cholerae* and *P. aeruginosa* use quorum sensing to control expression of virulence factors [33, 34]. Moreover, the siderophores of *P. aeruginosa* are also virulence factors, facilitating growth of the pathogen in infections [11, 16, 19, 33–38].

### 2.1.1 Bacterial Cooperation and the Problem of Cheating

Bacterial cooperation often relies on secreted factors that generate benefits for all members of the community, so called public goods [39]. While cooperation is a crucial part of the bacterial life, it is at the risk of being exploited by non-cooperating individuals, which readily evolve, and reap the benefits from cooperation without contributing to its costs. In the literature, they are referred to as cheaters [4, 19, 39–44]. By not contributing to a costly cooperative behaviour, these cheaters can allocate more resources into growth and are therefore favoured by natural selection [44–46]. This leaves us with the central question: if cooperation is seemingly not favoured by natural selection, why is cooperation still a ubiquitous feature in the bacterial world [47]? There have to be mechanisms that stabilizes bacterial cooperation in the presence of cheating individuals and indeed such mechanisms have been described (for a more detailed discussion see chapter 5 and [5]).

For example, the viscosity of the environment is predicted to greatly modulate the success of cooperation [48–53]. When viscosity is high, diffusion rate of public goods and therefore shareability is reduced. Hence, with increasing viscosity, the benefit for cheaters is reduced [53]. Viscosity can reduce the social aspect (sharing and cheating) of pyoverdine cooperation, but it can, however, not be abolished [54]. Additionally, molecular and regulatory properties of the cooperative trait can also help to maintain cooperation [43, 55]. By fine-tuning the regulation of siderophore production (see section 2.3.3), combined with the ability of *P. aeruginosa* to recycle and reuse its siderophores multiple times, the molecular and regulatory properties can shape the selection for siderophore-cooperation by maximizing the efficiency of siderophore production [53, 56, 57].

## 2.2 Quorum Sensing

Quorum sensing (QS) is one of the best studied cooperative behaviours in bacteria. QS is a communication tool for bacteria to coordinate population wide phenotypes and has first been discovered in the marine bacterium *Vibrio fischeri* in 1977 [58–60]. Since then QS has been investigated thoroughly on the social, mechanistic and genetic level [61–66]. Although the genetic components and regulatory pathways differ between species, the principle of QS is similar in quorum-sensing bacteria [67–70]. Cells secrete small signalling molecules into the environment, which can bind to specific receptors. When cells proliferate, signal molecules get more abundant and more molecules bind to the receptor. When the signal reaches a threshold, QS-controlled genes are up- or downregulated [60, 71]. QS is a cooperative trait, helping the community to optimally adapt to an environment and to regulate their phenotype [72]. QS cheaters, which either do not produce the signal, or are blind to it, can be observed regularly [73–75].

QS can control a variety of traits. In *Streptococcus pneumoniae*, QS acts like an on-off switch, facilitating the switch to competence (ability to take up exogenous DNA) and a fast reversion to the original behaviour [71]. In *Bacillus subtilis* two antagonistically working QS-systems allow to choose between two mutually excluding lifestyles (being competent or sporulation) [76, 77].

### Quorum Sensing and Virulence

QS often contributes to the virulence of pathogens [15]. For example, *V. cholerae* uses its QS-system to control expression of virulence factors. It promotes virulence factor production when cell numbers are low and downregulates virulence, when signalling molecules get more abundant (due to growth) [11, 34, 35]. In *P. aeruginosa*, QS controls the expression and secretion of virulence factors (among other traits) in later growth phases. Under the control of QS are:

proteases, like LasA and LasB [36], Exotoxin A (a toxin inhibiting elongation factor-2 in eukaryotic cells), pyocyanin and hydrogen cyanide [16]. *P. aeruginosa* requires QS in order to infect and colonize cystic fibrosis (CF) lungs [33].

## 2.3 Siderophores

Siderophores are small, secondary metabolites secreted by microbes to overcome iron starvation in environments where this essential nutrient is limited [25]. This is usually the case under physiological conditions, where iron is present in its insoluble ferric form or within a host, where ferric iron is actively withheld by the immune system to hinder bacterial growth [20, 25, 56, 78]. To date more than 500 different siderophores have been described in bacteria, yeasts and fungi [25]. Siderophores have a high stability constant<sup>1</sup> range ( $K_s = 10^{22}$  to  $10^{50}$ ) and can sequester iron from various sources like the iron binding protein transferrin [79].

### 2.3.1 The Siderophores of *Pseudomonas aeruginosa*

*P. aeruginosa* is capable of producing two different siderophores, namely pyoverdine and pyochelin [56, 80], with pyoverdine being the dominant siderophore system, as its affinity to iron ( $K_f = 10^{24} \text{ M}^{-1}$ ) [80] is much higher than the one of pyochelin ( $K_f = 10^5 \text{ M}^{-1}$ )<sup>2</sup> [56]. Other bacteria also have multiple siderophore systems. For example, *Escherichia coli* can produce enterobactin [81] and aerobactin [82, 83], while *Burkholderia cenocepacia* secretes ornibactin and pyochelin [84–86]. In bacteria multiple (sometimes redundant) systems are not uncommon, but it is still discussed why bacteria possess multiple siderophore

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<sup>1</sup>The stability constant  $K_s$  measures the strength of the interaction between two reagents, forming a complex.

<sup>2</sup>The binding constant  $K_f$  describes the affinity between a ligand and a protein.



systems. For *P. aeruginosa* it was suggested that pyochelin, since it is metabolically cheaper, is predominantly used in environments where iron limitation is less severe [87].

Once secreted, pyoverdine can be taken up by any cell with a compatible receptor and therefore it is a cooperative trait. Pyoverdine producing individuals can be exploited by non-producers. Such pyoverdine non-producing individuals readily evolve under shaken conditions [4, 5, 88], within hosts [19, 89, 90] and in the environment (Butaite E., Wyder S., Baumgartner M. and Kümmerli R., 2017, accepted). These non-producers benefit from the pyoverdine produced by others, without paying their share and thus they save resources. These cheaters can outcompete cooperating strains [4, 40, 53, 91].

### 2.3.2 Pyoverdine and Virulence

In infections iron is actively withheld by immune system to deprive bacteria of this essential nutrient. It is bound to iron binding proteins like transferrin, lactoferrin or heme<sup>3</sup>, but pyoverdine can scavenge iron from these proteins and is therefore secreted by *P. aeruginosa* to acquire sufficient quantities of iron [23, 25, 92–94]. Pyoverdine is an important virulence factor in acute infections, since pyoverdine deficient strains display reduced levels of virulence in various animal models [22, 23, 37, 94–100]. However, in chronic infections the ability of *P. aeruginosa* to produce pyoverdine is often reduced or lost [98, 101, 102].

Anderesen et al. [90] suggest that the loss of pyoverdine in chronic infections is not due to adaptation to the host, but caused by social dynamics. Pyoverdine non-producer thrive on the pyoverdine produced by others, and eventually outcompete pyoverdine producers [90]. This hypothesis is supported by

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<sup>3</sup>Hemes are a group of iron carrying cofactors, most commonly recognized in hemoglobin. Among other functions, they are responsible for the transportation of diatomic gases, where the iron acts as a electron acceptor.

the fact, that these pyoverdine non-producer retain their ability to express the pyoverdine receptor. Moreover, these cells upregulate the less effective heme uptake system to compensate for the loss of pyoverdine [103]. But, since these mutations (loss of pyoverdine and upregulation of heme uptake systems) happen on a timescale of years, they might not be causally linked. Furthermore, this hypothesis assumes that pyoverdine is freely diffusing within the infected compartments, a question we tackle in chapter 5. Therefore, more thorough experimental examination is necessary to validate this hypothesis.

Both, pyoverdine and pyochelin are important in infections [23] (details in section 2.3.2), this thesis, however, solely focuses on pyoverdine.

### **Pyoverdine Facilitates Expression of Virulence Factors**

Pyoverdine has a double role in infections. It is an iron chelator, but it can also act as a signalling molecule for virulence factors [21, 103, 104]. The pyoverdine regulator PvdS (see section 2.3.3) controls (directly or partially) the expression of two other virulence factors, namely Protease IV and Exotoxin A [16, 21, 105–107]. Protease IV (*piv*) is directly controlled by PvdS [106] and upregulation of pyoverdine could simultaneously lead to an increased expression of Protease IV. This protease might work synergistically with pyoverdine, by cleaving iron-loaded proteins (e.g. transferrin) and therefore freeing up iron, which becomes available for pyoverdine driven uptake [106].

Furthermore, the pyoverdine regulator (PvdS) partially controls the transcriptional regulator ToxR, which directly regulates the expression of Exotoxin A (ETA) [21, 105]. ToxR has a second promoter-site that is likely responding to QS [16]. Other factors influencing expression of ETA are cation concentration, temperature and oxygen levels [108–110]. ETA is the most toxic virulence factor in *P. aeruginosa* inhibiting the eukaryotic elongation factor-2 and thereby causing cell death [111, 112]. The regulatory link between pyoverdine, ETA and Protease IV

suggest that iron depletion plays a role in their expression. The functional role of this link is, however, still obscure.

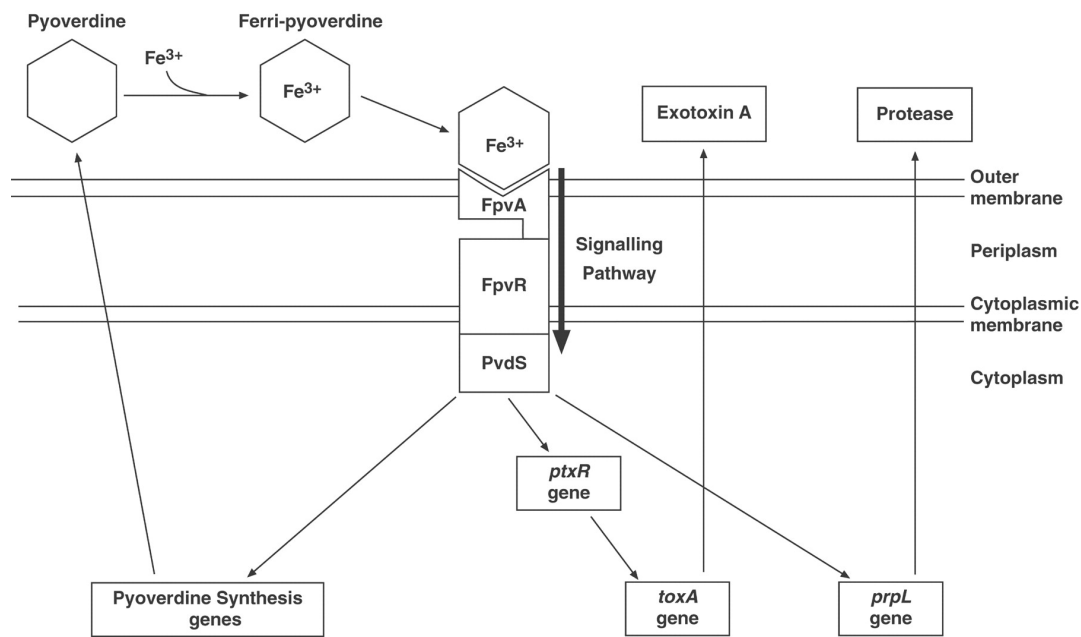
### 2.3.3 Regulation of Pyoverdine-Expression

Pyoverdine is a metabolically costly metabolite and therefore needs precise regulation to optimize costs and benefits of its production. Its regulation involves negative and positive feedback loops (see figure 1) and is governed by two central regulators, namely FUR (Ferric Uptake Regulator) and the alternative (extracytoplasmic<sup>4</sup>) sigma factor PvdS [56]. If iron levels in the cytoplasm are sufficient, FUR builds a complex with Fe<sup>2+</sup>, which then binds to the *pvdS*-promoter site, thereby inhibiting its transcription. When iron gets scarce, the Fe-FUR complex is dissolved and can no longer inhibit transcription of PvdS. Depending on growth phase, 582 to 720 copies of PvdS are present in the cytoplasm [113, 115]. PvdS in turn, promotes the expression of pyoverdine, which is produced in the cytoplasm (non-ribosomal peptide synthesis) and matured in the periplasmic space [116, 117]. Pyoverdine is subsequently secreted by an ATP depended efflux pump, consisting of an inner membrane protein (*pvdT*), a periplasmic adaptor protein (*pvdR*) and outer membrane protein (*opmQ*) with a  $\beta$ -barrel domain inserted in the outer membrane and a large periplasmic extension [118]. This efflux pump secretes newly synthesized pyoverdine, as well as pyoverdine that was previously taken up by the cell [118]. Active uptake of ferri-pyoverdine is followed by transport into the periplasmic space via a TonB system<sup>5</sup>. In the periplasm, iron is released from pyoverdine by reduction from Fe<sup>3+</sup> to Fe<sup>2+</sup> and transported into the cytoplasm, while pyoverdine is repeatedly secreted [56].

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<sup>4</sup>Extracytoplasmic sigma factors usually react to environmental cues, governing expression of extracytoplasmic functions [113, 114].

<sup>5</sup>TonB-dependent transporters are outer membrane proteins that bind and transport e.g. iron loaded siderophores, which requires energy in the form of proton motive force [119].



**Figure 1:** Scheme of the pyoverdine regulation pathway. The ferri-pyoverdine complex binds to the specific receptor FpvA, transmitting a signal through the cell membrane, promoting transcription of PvdS (via FpvR) and therefore the transcription of pyoverdine. Additionally, it can promote production of *piv* (Protease IV) and *ptxR* (increases *toxA* expression by four to fivefold [108]). Additionally, PvdS can regulated *toxR*, the regulator of ETA (pathway not shown in this figure). Figure from [104].

Since pyoverdine is stable and functional over (at least) 48 hours, pyoverdine recycling reduces the costs of pyoverdine production [43].

### Uptake of Iron Loaded Pyoverdine Triggers Pyoverdine Production

FpvR is an anti-sigma factor that, in the absence of pyoverdine, directs PvdS into proteolytic pathway, therefore reducing pyoverdine production [113]. Upon binding of iron loaded pyoverdine (ferri-pyoverdine) to the specific pyoverdine receptor FpvA, a signal is transmitted through the cell membrane. The FpvA-signal suppresses the anti-sigma activity of FpvR, triggering the activation of PvdS and the release of membrane-bound PvdS to positively regulate expression of pyoverdine [56, 104, 120, 121]. Additionally, FpvA promotes the transcription of the extracytoplasmic sigma-factor FpvI, which upregulates the

production of the pyoverdine receptor (figure 1) [21, 56]. This regulation cascade is only triggered by the binding of iron-loaded pyoverdine [56, 104, 113]. Consequently, upon binding of iron-loaded pyoverdine, the expression of pyoverdine and its receptor is upregulated until iron supply is sufficient, which leads to a negative feedback by FUR, inhibiting *pvdS*-transcription, eventually causing a shutdown of pyoverdine production. Thus, pyoverdine positively regulates its own and the expression of its receptor. When iron loaded pyoverdine binds to the receptor, it can cause a cascade of transcriptional and regulatory changes (pyoverdine cluster, pyoverdine receptor, Protease IV and ETA), ultimately contributing to the virulence of *P. aeruginosa* [56].

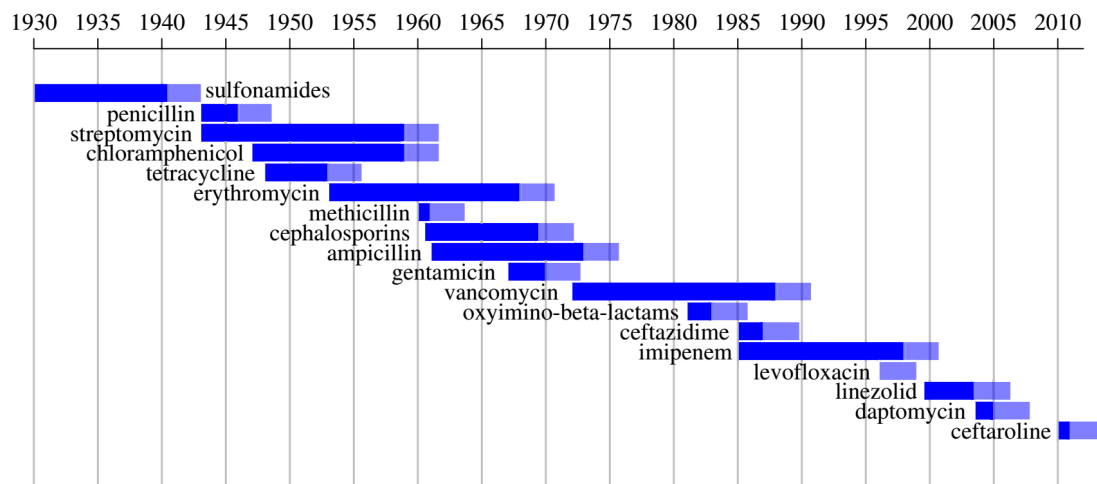
## 2.4 New Approaches to Fight Multi-Drug-Resistant Pathogens

### 2.4.1 The Problem of Antibiotic Resistance

Alexander Fleming, who discovered penicillin in 1928, predicted in his Nobel lecture (1945) that bacteria can develop resistance to penicillin quite easily (Alexander Flemming: Nobel Lecture, December 1945 <sup>6</sup>). Over 60 years later, we realised that bacteria not only develop resistance to penicillin, but to all classes of antibiotics (figure 2), even to antibiotics where resistance was thought to be impossible (e.g. vancomycin [122, 123] and to last resort antibiotics like carbapenems [124]). The use of antibiotics has become unsustainable and physicians are running out of antibiotics they could use to cure infections caused by multi-resistant bacteria [125]. It is often postulated that a limitation of antibiotic usage will help to maintain their efficacy [126–129]. Though it is crucial to use

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<sup>6</sup>[https://www.nobelprize.org/nobel\\_prizes/medicine/laureates/1945/fleming-lecture.pdf](https://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-lecture.pdf), accessed 17.03.2017, 13:30



**Figure 2:** Time line for different classes of antibiotics from introduction to loss of efficacy. The bars indicate the introduction and subsequent evolution of resistance. The faded region denotes the time between the first report of resistance to the complete loss of efficacy. Adapted from [137]. Original data from [138].

antibiotics efficiently, we may have passed the threshold where solely limiting usage will improve the situation [130]. While antibiotic resistance becomes an increasing risk for public health, the development and release of new antibiotic agents has almost stalled [131, 132]. Incentives to develop new classes of antibiotics are low due to the short expected lifespan [133]. Support for development of new classes of antibiotics is crucial, but every new agent, that is based on the principal of killing bacteria or hindering their growth, is potentially doomed to become ineffective over time as evidenced by the rapid rise and spread of antibiotic resistance by de-novo acquiring or horizontal gene transfer [122, 123, 134–136]. Eventually nosocomial pathogens like *Acinetobacter baumannii*, *P. aeruginosa* or *Enterobacteriaceae* (which have been declared by the WHO, to be the most dangerous bacterial pathogens<sup>7</sup>) will acquire resistance.

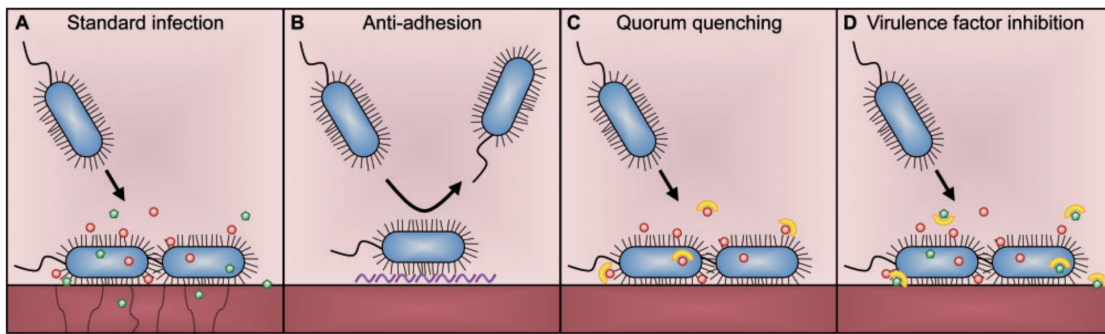
It is apparent that we need new concepts to fight infections, caused by multi-

<sup>7</sup>[http://www.who.int/medicines/publications/WHO-PPL-Short\\_Summary\\_25Feb-ET\\_NM\\_WHO.pdf](http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf), accessed 09.03.2016, 10:30

resistant bacteria. One strategy is to improve hygiene standards in hospitals and to test disease causing pathogens for their susceptibility to antibiotics to ensure that the administration of antibiotics is as effective as possible [139]. Furthermore, we could try to find novel strategies how to get along with the drugs we have at our disposal. Such approaches involve mixing (combination therapies) and cycling of drugs (alternating antibiotics). It is believed that bacteria should have difficulties to simultaneously develop resistance to two or more antibiotics with different modes of action [140, 141]. But combination therapies and antibiotic cycling are at the risk of becoming ineffective if the wrong antibiotics are applied, since bacteria could activate mechanisms that confer resistance to multiple drugs.

### **2.4.2 Anti-Virulence Treatments**

The reason why bacteria develop resistance is simply natural selection, which favours only those strains that are capable of surviving the deleterious effects of antibiotics [142]. Hence, the high rate of resistance in nosocomial pathogens is owned to the high killing potential of antimicrobial agents [134]. Taking this into consideration, new therapeutic strategies should involve approaches that escape the strong selection pressure of antibiotics, thereby minimizing the evolutionary response [134, 142]. It was suggested that, instead of targeting vital cell mechanism, we could reduce the virulence of pathogens by disarming them [143–146]. Such treatments (figure 3) have been coined with the term "anti-virulence treatments" and currently there is a lot of interests to find agents that target bacterial virulence factors [18, 137, 142, 144, 145, 147–149]. Additionally, it was proposed that such anti-virulence treatments can become evolution proof (for details see section 2.5) [96, 142]. A treatment is evolution proof, if bacteria cannot develop resistance to it.



**Figure 3:** (A) In a classical infection, bacteria adhere to host tissue, using their flagella and pili. They then secrete quorum-sensing molecules (red dots) to coordinated virulence factor (green dots) expression such as siderophores, tissue degrading enzymes or proteases. All named traits can be targets for potential anti-virulence treatments. (B) They could prevent adhesion, (C) silent microbial communication by quenching quorum sensing molecules (quorum quenching), (D) or inhibit/quench bacterial toxins, proteases or other virulence factors. Image credits: [142]

### Examples of Anti-Virulence Treatments

An example for an anti-virulence treatment are phosphonosulphonates, which reduce the virulence of *Staphylococcus aureus*. Phosphonosulphonates inhibit the production of staphyloxanthin, a bacterial anti-oxidant pigment that normally protects *S. aureus* from neutrophil-based killing and by inhibiting it, the cells become exposed to the immune system [150].

In another approach, Henry and colleagues elegantly inhibit toxins of the CDC-family (cholesterol-dependent cytolysins), by constructing artificial liposomes [151]. These toxins are produced by a wide range of gram positive pathogens, including *Clostridium*, *Streptococcus*, *Listeria* and *Bacillus* [152], in order to form pores into the cell wall of host cells [151], thereby facilitating the infection. They constructed individual artificial liposomes, which could effectively bind various toxins during infections. By using a mixture of these liposomes, the authors were able to inhibit various toxins produced by staphylococcal pathogens and treat, otherwise fatal infections in a mouse model [151].

In cystic fibrosis infections, *P. aeruginosa* forms biofilms, which are associated with antibiotic resistance and protection from the immune system [30, 153, 154].



Consequently, biofilms are an attractive target for treatment of *P. aeruginosa* and other biofilm making pathogens [155–160]. These "anti-biofilm"-treatments could either work by interfering with biofilm-regulation or by destroying the exopolysaccharides of the biofilm. By doing so, bacteria are more accessible to the immune system and antibiotic treatment and infections can be cleared more easily [161].

Moreover, we can inhibit siderophores of pathogens to undermine iron supply of the bacteria and consequently reducing their pathogenicity. In chapter 3 we propose gallium as an anti-virulence treatment that inhibits the siderophore of *P. aeruginosa* [162, 163]. More examples for potential targets of anti-virulence treatments can be found in table 2.2 at the end of this chapter.

### **Reducing Virulence by Interfering with Quorum Sensing**

Another attractive target is the QS system of pathogens. Manipulating QS would reduce expression of multiple virulence factors simultaneously. In *V. cholerae* QS controls the expression of virulence factors at early stages of the infection. When the QS-signal increases (due to growth), virulence factors production is suppressed (see section 2.2). Addition of QS-signalling molecules could therefore promote a non-virulent state [164].

In *P. aeruginosa*, QS regulates the expression of multiple virulence factors like proteases, pyocyanin, Exotoxin A, etc. [16, 36]. By silencing QS, expression of these virulence factors would be abolished, making *P. aeruginosa* infections more benign. This approach has been described as "quorum quenching". The quorum sensing process is basically divided into three steps: (i) production of the signal, (ii) accumulation of the signal and (iii) binding to the receptor. Quorum quenching compounds can interfere with all three steps. For example one could attempt to degrade the signalling molecules extracellularly. Many bacteria and other organisms secrete metabolites to degrade signalling molecules

from other species [165–168] and also our immune system is capable of destroying signalling molecules produced by the LasR-system of *P. aeruginosa* [169]. Three categories of enzymes are known to be able to degrade AHLs (produced by *P. aeruginosa*): acylases, lactonases, oxidoreductases. Each of them has been shown to target quorum sensing molecules produced by the LasR- and RhlR-system of *P. aeruginosa* [59]. Moreover we can make use of e.g. furanones, which interfere with receptor binding of QS-signalling molecules, thereby inhibiting accumulation of the signal and consequently the expression of QS-controlled traits [170–173].

### 2.4.3 The Evolution of Resistance to Anti-Virulence

#### Treatments

Antibiotics often require uptake of the drug by the cell. Among other mechanisms, multidrug efflux pumps are part of the innate or acquired resistance to antibiotics and are upregulated in the presence of antibiotics [174–181]. Put simply, everything which enters the cells could be pumped out using such efflux pumps. By administering antibiotics, the cells are exposed to strong selection pressure and e.g. upregulating the efflux pump results in strong positive evolutionary feedback, namely the survival of the cell [144]. The same is true for treatments that target virulence factors within cells. Resistance can be acquired by classical multi drug resistance mechanisms (table 2.1).

Anti-virulence treatments aim to reduce selection pressure on bacterial cells, making resistance less likely. But is this really the case? Phosphonosulphonates (section 2.4.2) expose *S. aureus* to the immune system and therefore to the removal from the infection site. Hence, they impose a strong selection pressure on the bacteria, making resistance more likely [142]. Furthermore, phosphonosulphonates have to enter the cell, making it easier for *S. aureus* to develop resistance.

In studies [182] and [183] the resistance to quorum quenching compounds (fura-

Routes to resistance	Drug must enter the cell	Drug works outside the cell	Pyoverdine is inhibited by gallium
Preventing drug entry	yes	no	no
Increasing drug efflux	yes	no	no
Target modification	yes	yes	Yes
Drug degradation	yes	yes	no
Switch to alternative virulence factors	yes	yes	yes

**Table 2.1:** Possible routes to resistance for treatments that target virulence factors within the cell and outside of the cell and for gallium, the anti-virulence treatment described in chapter 3.

nes) is described. Furanones block the receptor for quorum sensing molecules within the cell (AHLs bind to the receptor intracellularly to form a multimer that can bind DNA to regulate genes [33, 184]). Hence they could be subjected to resistance mechanisms like efflux pumps. Moreover, resistance to enzymes that inhibit AHLs outside of the cell or disrupt biofilms, could be acquired by extracellular degradation of these compounds. Consequently, in terms of resistance, such agents are more like antibiotics and therefore may be equally prone to resistance.

The anti-virulence agent we used in chapter 3 also reduces growth of the pathogen and thereby reduces virulence [96]. However, Garcia-Contreras et al. (2013) claim to find resistance to gallium (the anti-virulence agent we used in) [185]. If resistance to anti-virulence agents is seemingly possible, how can we then make anti-virulence agents evolution proof?

## 2.5 Evolution Proof Drugs

Anti-virulence treatments are predicted to be evolution proof if (i) they have marginal fitness effects on the pathogen, (ii) target a secreted virulence factor

and (iii) the virulence factor is cooperatively shared between individuals [142]. First, inhibiting a virulence factor with marginal fitness effects reduces the evolutionary pressure to become resistant [142]. Second, by targeting a secreted virulence we can circumvent some resistance mechanisms (see table 2.1; details in section 2.5.1) [186]. And third, by targeting a cooperatively shared virulence factor, we can disconnect the rise of a resistance mutant and its spread in the community (for details see 2.5.2). Hence, observing the rise of a resistant phenotype (as in [185]) does not automatically imply that resistance will spread within the community [187].

### **2.5.1 Targeting a Secreted Virulence Factor**

So far, the term virulence factor was used as a generic term for factors which facilitate infections e.g. help the bacteria to overcome the host's immune response or to proliferate at the infection site. However, it is crucial to distinguish between virulence factors that are associated with the cell and virulence factors which are secreted into the environment. Treatments that inhibit secreted virulence factors do not enter the cell, hence they can escape some resistance mechanisms like efflux pumps or preventing drug entry (see table 2.1) [142].

In section 3 we quenched the virulence factor pyoverdine by using gallium. Pyoverdine cannot distinguish between iron and gallium, but binds preferentially to the later, rendering it useless as an iron delivery system [162, 163]. By quenching pyoverdine we inhibit a secreted virulence factor and potential routes to resistance are limited (table 2.1). However, as described in [185], individual cells can become resistant to gallium.

Consequently, treatments that target secreted virulence factors minimize, but do not abolish the possibility of resistance development and are therefore not evolution proof.

### 2.5.2 Targeting a Cooperatively Shared Virulence Factor

It is crucial to distinguish between virulence factors that benefit only the producer and virulence factors that generate benefits for all cells in a collective [144]. Many bacterial virulence factors belong to the later class [188]. Shared virulence factors, scavenge nutrients (e.g. siderophores) and digest or liberate resources (e.g. proteases, elastases) to promote growth at the infection site [5, 22, 89, 188, 189]. The production and secretion of these virulence factors is costly, but benefits all compatible cells in the vicinity. By inhibiting such a virulence factor, the pathogen becomes a phenotypic non-producer and thus will exploit any resistant phenotype, e.g. by resuming cooperation (see section 2.1.1) [190]. Hence, this resistant phenotype would not be favoured by natural selection and cannot spread. Although anti-virulence treatments aim to minimize the chance that bacteria develop resistance, it is possible that a resistant phenotype arises (as evident in [185]). But by targeting a collectively shared virulence factor, we can exploit social dynamics and create an environment where resistance is selected against, hence the anti-virulence agent can become evolution proof.

In our previously mentioned study (chapter 3), we quenched the siderophore pyoverdine by using gallium. We argued that the likelihood of resistance to gallium is low and that gallium might be a good candidate for an evolution proof drug [191]. In chapter 3 (table 1) we discuss in detail potential resistance mechanisms against gallium and if these mechanisms can facilitated resistance of the community.

## 2.6 Aims of this Thesis

In this thesis, I tackled three objectives. In the first chapter (3), we will test the anti-virulence agent gallium for its potential to reduce the virulence of *P. aeruginosa*. Gallium inhibits pyoverdine irreversibly, reducing iron uptake and therefore the potential of *P. aeruginosa* to cause damage to the host. To test our

hypothesis, we will use an insect model and record the survival of larvae in the presence or absence of gallium. Moreover, we propose that gallium is a promising candidate for an evolution proof treatment and therefore we will assess its potential for resistance development compared to conventional antibiotics.

In chapter (4) we hypothesise that administration of gallium can have complex consequences for the pathogen, its regulatory network and the host. To test our hypothesis, we will use the same insect model as above and vary the availability of the virulence factor pyoverdine. We will assess virulence, pathogenic growth (*in-vivo*), expression of virulence factors (which are regulatorily linked to pyoverdine) and the hosts response.

In the third part of the thesis, I will address a different question. Bacterial cooperation has been thoroughly investigated over the last decade, but mainly in liquid cultures across millions of cells. Predictions derived from such experiments may have only limited significance for environments where diffusion of public goods is reduced (higher viscosity) and cell number is strongly limited, e.g. during infections. The objectives above, however, assume that under such conditions, cooperation is a crucial part of pathogenicity. Therefore, we will verify this assumption by studying cooperation of surface attached bacteria at the level of single cells by using fluorescence microscopy. We will track the effect of cooperation when cell numbers and diffusivity of pyoverdine is severely limited. By using fluorescent reporter strains, we aim to measure investment of bacteria into pyoverdine production in the presence and absence of pyoverdine non-producers. Moreover, by assessing the natural fluorescence of pyoverdine in pyoverdine non-producers we want to investigate the shareability of pyoverdine in highly structured environments. From experiments in batch culture we know that pyoverdine cheating boosts growth of non-producer at the cost of cooperating individuals. To establish the physical boundaries of pyoverdine sharing, we will compete producers and non-producers and manipulate the distance between the competitors and the viscosity of environment.

Virulence Factor	Role at Infection side	Possible treatment
Siderophores	Iron-scavenging molecules [27]	gallium [96, 163]
Adhesion inhibitors	Adhesion to other cells or surfaces	sortase A inhibits adhesion of <i>S. aureus</i> [192, 193]
Exopolysaccharide	Providing structure for growth and protection ([194, 195])	Inhibiting or destroying biofilms e.g. [196]
Shiga toxins	Breaking down host tissue [197]	Urtoxatumab [198]
Proteases	Extracellular protein digestion [199]	NA
Bacterial toxins and toxin co-regulated factors	Inflammation, releasing nutrients	Artificial liposomes [151] and Vir-satin [200]
Manipulating QS-systems	Interfering with synthesis, accumulation or receptor binding of signaling molecules	Overview in [59]

**Table 2.2:** List of potential targets for anti-virulence treatments and (if available) possible agents that inhibit these virulence factors.

## CHAPTER 3

### **Gallium-Mediated Siderophore Quenching as an Evolutionarily Robust Antibacterial Treatment**

This research was originally published in *Evolution, Medicine and Public Health*

**Ross-Gillespie, A.\*, Weigert\*, M., Brown S.P, and Kümmerli R.**, 2014, Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment, *Evolution, Medicine, and Public Health* 2014 (1): 18-29.

doi: 10.1093/emph/eou003

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# Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment

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Received 19 December 2013; revised version accepted 24 January 2014

## ABSTRACT

**Background and objectives:** Conventional antibiotics select strongly for resistance and are consequently losing efficacy worldwide. Extracellular quenching of shared virulence factors could represent a more promising strategy because (i) it reduces the available routes to resistance (as extracellular action precludes any mutations blocking a drug's entry into cells or hastening its exit) and (ii) it weakens selection for resistance, as fitness benefits to emergent mutants are diluted across all cells in a cooperative collective. Here, we tested this hypothesis empirically.

**Methodology:** We used gallium to quench the iron-scavenging siderophores secreted and shared among pathogenic *Pseudomonas aeruginosa* bacteria, and quantitatively monitored its effects on growth *in vitro*. We assayed virulence in acute infections of caterpillar hosts (*Galleria mellonella*), and tracked resistance emergence over time using experimental evolution.

**Results:** Gallium strongly inhibited bacterial growth *in vitro*, primarily via its siderophore quenching activity. Moreover, bacterial siderophore production peaked at intermediate gallium concentrations, indicating additional metabolic costs in this range. *In vivo*, gallium attenuated virulence and growth—even more so than in infections with siderophore-deficient strains. Crucially, while resistance soon evolved against conventional antibiotic treatments, gallium treatments retained their efficacy over time.

**Conclusions:** Extracellular quenching of bacterial public goods could offer an effective and evolutionarily robust control strategy.

**KEYWORDS:** antivirulence therapy; public good quenching; resistance; experimental evolution; *Pseudomonas*

### 3 Gallium-Mediated Siderophore Quenching as an Evolutionarily Robust Antibacterial Treatment

#### INTRODUCTION

Like all organisms, pathogens acquire genetic mutations, and, in time, even 'pure' cultures will inevitably come to harbor mutant lineages. Such genetic variability can make some pathogen variants less sensitive to therapeutic interventions than others, and under strong or sustained therapy, these resistant variants will have a selective advantage and will come to predominate over more susceptible variants. Consequently, the therapy will lose efficacy [1, 2]. To avoid this situation, we can try to prevent resistant variants from arising and/or from spreading [3]. To prevent resistance arising, we could attempt to reduce mutation supply, through limiting effective population size or by employing interventions with specialized modes of action where relatively few 'routes to resistance' are possible. To prevent spread, meanwhile, we must aim to minimize fitness differences across individual pathogens. Killing every individual, the conventional antibiotic strategy, could certainly quash fitness evenly, but this is difficult in practice and whenever incomplete gives resistant pathogens a strong relative fitness advantage. 'Antivirulence' treatments, meanwhile, ostensibly disarm but do not harm pathogens, such that resistant variants should benefit little relative to susceptibles [4]. However, traits that affect virulence but not fitness are rare, and the label 'antivirulence' is used liberally, even for interventions that yield substantial fitness differences among pathogens [4]. A final way to minimize fitness differences is to target pathogens' collective traits, where costs and benefits are widely shared. For instance, many virulence-related bacterial exoproducts are also public goods (PGs) [5]. Under PG-quenching therapy, any mutations allowing PGs to build up again should benefit both resistant and susceptible individuals alike, which would hinder the spread of resistance [1, 6–8].

To illustrate why this matters, let's consider a specific example. Quorum quenching (QQ), which disrupts the cell-to-cell communication [quorum sensing (QS)] [9] underlying a wide range of collectively expressed virulence traits, is a PG-targeting 'antivirulence' therapy regarded as a promising alternative to conventional bacteriocidal or bacteriostatic treatments [10, 11]. However, early enthusiasm for QQ has been tempered recently by reports that bacteria can quite readily evolve resistance to such treatments [12–14]. Set against our framework, this is unsurprising: first, QQ interventions

frequently involve intracellular action, against which many potential resistance-conferring adaptations could arise (e.g. modified membrane properties to block a drug's entry into a cell, or upregulated efflux pumps to hasten its exit [15]). Second, QS regulates not only PGs but also certain essential private goods [16], giving QQ resistants substantial personal benefits over susceptibles—and therefore a means to spread. For maximal evolutionary robustness, we need therapies where resistance mutations are unlikely to arise in the first place (e.g. extracellular action restricts potential routes to resistance) and are also unlikely to spread, because fitness differences between resistant and susceptible pathogens are minimized. The latter should be the case when collective traits are targeted, because fitness consequences are shared across many individuals. Of course, the extent and evenness of this sharing will depend on the relatedness and spatial structure of the pathogen population and the diffusive properties of the environment, and these factors would also need to be considered during therapy design [3].

In this study, we investigate—in a test case—the hypothesis that extracellular PG quenching is an effective and evolutionarily robust strategy for pathogen control. The PG trait we target is siderophores, important exoproducts whose regulation is not linked to any exclusively private goods. Siderophores are diffusible molecules with a high affinity for ferric iron ( $\text{Fe}^{3+}$ ) and are secreted by most bacteria to scavenge this important but generally bio-unavailable form of iron from their environment or, in the case of pathogens, from their host's own iron-chelating compounds [17]. Once loaded with  $\text{Fe}^{3+}$ , siderophores are taken up by producer cells—or other nearby individuals equipped with appropriate receptors—stripped of their iron, and secreted once again into the environment [18]. Although their primary function may be to scavenge iron, siderophores also bind, with varying success, several other metals [19, 20]. Among these, gallium is the closest mimic of iron.  $\text{Ga}^{3+}$  and  $\text{Fe}^{3+}$  ions have very similar ionic radii and binding propensities but, crucially, while  $\text{Fe}^{3+}$  reduces readily,  $\text{Ga}^{3+}$  does not [19].  $\text{Ga}^{3+}$  therefore cannot replace iron as a co-factor in redox-dependent enzymes. We investigated the iron-mimicking effects of gallium on pyoverdine, the primary siderophore of *Pseudomonas aeruginosa* [21], a widespread opportunistic pathogen with a broad host range and, in humans, the cause of

notoriously persistent infections in immune-compromised tissues, cystic fibrosis lungs and in association with implanted devices [22]. Pyoverdine, which plays an important role in such infections [23, 24], binds gallium at least as readily as iron, and gallium-bound pyoverdine is of no use to iron-starved cells [19, 20]. Thus, even without entering the cell, gallium can reduce *P. aeruginosa* growth and biofilm formation by quenching local stocks of secreted pyoverdine and choking off iron supply [19, 25].

Below, we report our investigations into (i) gallium's *in vitro* interference with siderophore-mediated iron uptake and consequent effects on bacterial growth, (ii) gallium's *in vivo* effects on virulence and in-host bacterial growth and (iii) the potential for bacteria to evolve resistance against gallium treatment.

## METHODOLOGY

### Strains and media

*Pseudomonas aeruginosa* strains featured in our experiments included the wild-type strain PAO1 (ATCC 15692), the siderophore knock-out mutants PAO1 $\Delta$ *pvdD* and PAO1 $\Delta$ *pvdD* $\Delta$ *pchEF* [26], provided by P. Cornelis, Free University of Brussels, Belgium, as well as versions of the above strains constitutively expressing GFP (PAO1-*gfp*, PAO1 $\Delta$ *pvdD*-*gfp*, chromosomal insertion: *attTn7::ptac-gfp*), and a version of PAO1 with a *pvdA-gfp* reporter fusion (PAO1*pvdA-gfp*, chromosomal insertion: *attB::pvdA-gfp*) [27], provided by P. K. Singh, University of Washington, USA. We also used the Rhl-quorum-sensing deficient mutant PAO1 $\Delta$ *rhlR*, provided by S. P. Diggle, University of Nottingham, UK. For overnight culturing, we used Luria Bertani (LB) medium, while for experimental assays we used CAA medium, supplemented with FeCl<sub>3</sub> where indicated to manipulate iron availability. LB was obtained pre-mixed from Sigma-Aldrich, Switzerland. Our standard CAA medium contained 5 g l<sup>-1</sup> casamino acids, 1.18 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.25 g l<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 µg ml<sup>-1</sup> human-apo transferrin, 20 mM NaHCO<sub>3</sub> and 25 mM HEPES buffer (all from Sigma-Aldrich).

### *In vitro* assays of growth and pyoverdine production

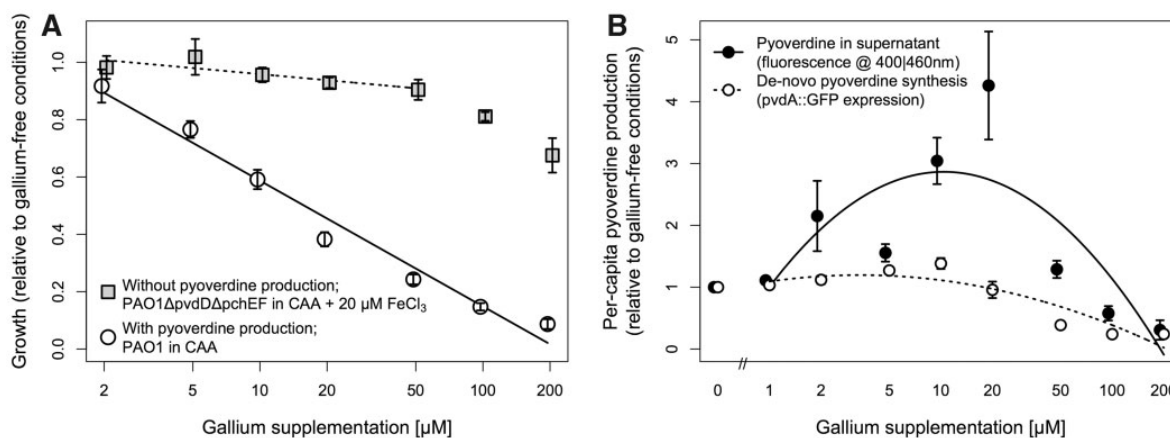
Overnight LB cultures (37°C, 180 rpm), washed and standardized for cell density, were diluted to 10<sup>-4</sup> then used to seed replicate cultures in CAA medium

supplemented with Ga(NO<sub>3</sub>)<sub>3</sub> (such that final Ga concentrations ranged from 0 to 200 µM), as well as complementary amounts of NaNO<sub>3</sub> to balance nitrate levels across treatments, and 20 µM FeCl<sub>3</sub> where iron-replete conditions were required (Fig. 1A). Growth assays were performed with 200 µl cultures in 96-well plates, for which optical density (OD) was tracked over 24 h at 37°C using a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland), with 15 min read intervals preceded at each read by 10 s of agitation. To assay pyoverdine production, we first grew PAO1 *pvdA-gfp* in 2 ml CAA static in 24-well plates in a 37°C incubator for 24 h, then centrifuged the cultures at 7000 rpm for 2 min to pellet the cells. From each culture, 200 µl of supernatant and, separately, the cell fraction resuspended in 200 µl 0.8% saline, were transferred to a new 96-well plate and assayed for OD at 600 nm and fluorescence (GFP in cell fraction: ex|em = 488|520 nm; pyoverdine in supernatant: 400|460 nm) [28]. Both fluorescence measures were standardized by OD at 600 nm. In a series of side experiments, we investigated potential biases associated with the use of optical measures as proxies for pyoverdine production (Supplementary Fig. S1). Data presented in Fig. 1B are corrected for these biases.

### Experimental infections

Infection assays were performed with final instar *Galleria mellonella* larvae, purchased from a local supplier, standardized for mass and general condition and stored at 4°C until use (within 3 days). A Hamilton precision syringe was used to deliver 10 µl inocula via a sterile 26s gauge needle introduced sub-dermally to a surface-sterilized area between the last pair of prolegs. Inoculations contained Ga(NO<sub>3</sub>)<sub>3</sub> diluted to different concentrations in 0.8% saline, with complementary concentrations of NaNO<sub>3</sub>, and, where specified, bacteria from overnight LB cultures (37°C, 180 rpm), standardized for cell density and diluted such that each 10 µl inocula contained ~25 CFU (*post hoc* counts of 12 inocula plated out to LB agar gave 95% CI of 19.41–31.76). Specifically, we tested the following Ga(NO<sub>3</sub>)<sub>3</sub> concentrations: 2.5, 10 and 50 µM ('LOW'; pooled together since their resulting virulence curves were not significantly different from one another), 500 µM ('MED') and 2500 µM ('HIGH'). Our 'Gallium only' treatment comprised various concentrations between 2.5 and 2500 µM, which again we pooled for statistical analyses because of similar effects

### 3 Gallium-Mediated Siderophore Quenching as an Evolutionarily Robust Antibacterial Treatment



**Figure 1.** Gallium affects *P. aeruginosa*'s *in vitro* growth and siderophore production. (A) Gallium suppresses growth particularly when pyoverdine is present, as shown here by comparing conditions with and without its production. Symbols and bars indicate means and 95% CIs of integrals of spline curves fitted through 24 h growth trajectories (OD at 600 nm) of 12 replicate cultures. (B) Pyoverdine, assayed using complementary approaches, is in each case upregulated at intermediate gallium concentrations. Symbols and error bars represent means and SEs of five replicates. Measures of pyoverdine from supernatant (filled circles) or *pvdA* expression from cell fractions (open circles) are in each case scaled by cell density (OD at 600 nm).

on survival. Post-injection, larvae were placed individually in randomly allocated wells of 24-well plates and incubated at 37°C. Survival was monitored hourly between 10 and 24 h, and larvae were considered dead once they no longer responded to tactile stimulation. Any larvae that began to pupate while under observation or died within the first 10 h post-injection (i.e. as a result of handling) were excluded from analyses ( $n = 23$ , 3.6%). To assay *in vivo* bacterial growth, we prepared our inocula with strains engineered to constitutively express GFP (see above), having previously established that constitutively expressed GFP signal could provide a reliable correlate of bacterial density under the conditions of this infection model (Supplementary Fig. S2). In each of six separate experimental blocks, and at each of four discrete timepoints, 3–4 randomly selected larvae per treatment were flash-frozen in liquid N<sub>2</sub> and manually powdered. Powdered larval homogenates were resuspended in 1 ml sterile H<sub>2</sub>O, vigorously shaken and then centrifuged at 7000 rpm for 2 min, whereafter the sample segregated into discrete phases. About 200 µl of the water-soluble liquid phase was extracted and assayed for GFP-fluorescent signal relative to control replicates (saline-injected larvae), using a Tecan Infinite M-200 plate reader. Given total larval volumes of ~1 ml, and assuming that ~20% of this volume might be hemolymph accessible to particles diffusing from a single injection site during the course of an acute

infection, we estimate that inocula gallium concentrations of 2.5–2500 µM would translate to in-host gallium concentrations of roughly ~0.05 to ~50 µM.

#### Experimental evolution

We compared the growth inhibitory effects of gallium versus the aminoglycoside, gentamicin (Gm), and the fluoroquinolone, ciprofloxacin (Cp)—two of several antibiotics recommended for clinical use against *P. aeruginosa* [29]. Concentrations were calibrated such that they reduced growth integrals over the initial 24 h to  $\leq 1/3$  that of untreated PAO1 WT cultures under the same growth conditions. For each of 12 days, a 96-well plate was prepared, comprising replicate 198 µl volumes of iron-limited CAA medium supplemented, according to a randomized layout scheme, with gallium, antibiotics or an equivalent volume of saline (see key in Fig. 3 for details of treatments used and their respective sample sizes). Day 1 cultures were initiated with 2 µl aliquots of a 10<sup>-3</sup> diluted overnight LB culture of PAO1 WT (37°C, 180 rpm), while for subsequent days, fresh plates were inoculated with 10 µl of undiluted culture from the corresponding wells of the previous day's plate, directly after it completed its growth cycle. Plates were incubated at 37°C, and cell density and pyoverdine fluorescence measures were recorded at 15 min intervals (with 10 s initial shaking) using a Tecan Infinite M-200 plate reader.

### Endpoint phenotypic assays

Prior observations [30] and our own reasoning (see Table 1) suggested that pyoverdine and pyocyanin could both affect the costs and benefits of iron uptake under gallium treatment. Anticipating that the experimental evolution described above might have induced changes in these traits, we performed phenotypic assays to compare cultures of our ancestral PAO1 WT, its descendent lines experimentally evolved in CAA with or without supplementation with 20  $\mu$ M Ga, and also two knock-out mutant strains which served as negative controls: PAO1 $\Delta$ *pvdD* (deficient for pyoverdine production) and PAO1 $\Delta$ *rhIR* (deficient for the Rhl-quorum-sensing system which regulates pyocyanin production [31]). Specifically, we inoculated 2 ml volumes of growth medium (either LB or CAA) with 20  $\mu$ l of  $10^{-3}$  diluted overnight LB culture and incubated at 37°C in static conditions. After 24 h, we measured OD 600, centrifuged at 7000 rpm for 2 min, then extracted 200  $\mu$ l aliquots of supernatant and assayed these for growth (OD at 600 nm) and levels of pyocyanin (using OD at 691 nm) [32] and pyoverdine (fluorescence at 400|460 nm), using a Tecan Infinite M-200 plate reader.

### Statistical analyses

All analyses were performed using R 3.0.0 [33]. Spline curves were fitted to time course growth data using the 'grofit' package [34]. Survival analyses were performed using the Surv package [35]. Although in the main text we compared survival curves using parametric Weibull models, we also repeated all analyses using Cox proportional hazards regressions, and obtained qualitatively comparable results in all cases.

## RESULTS

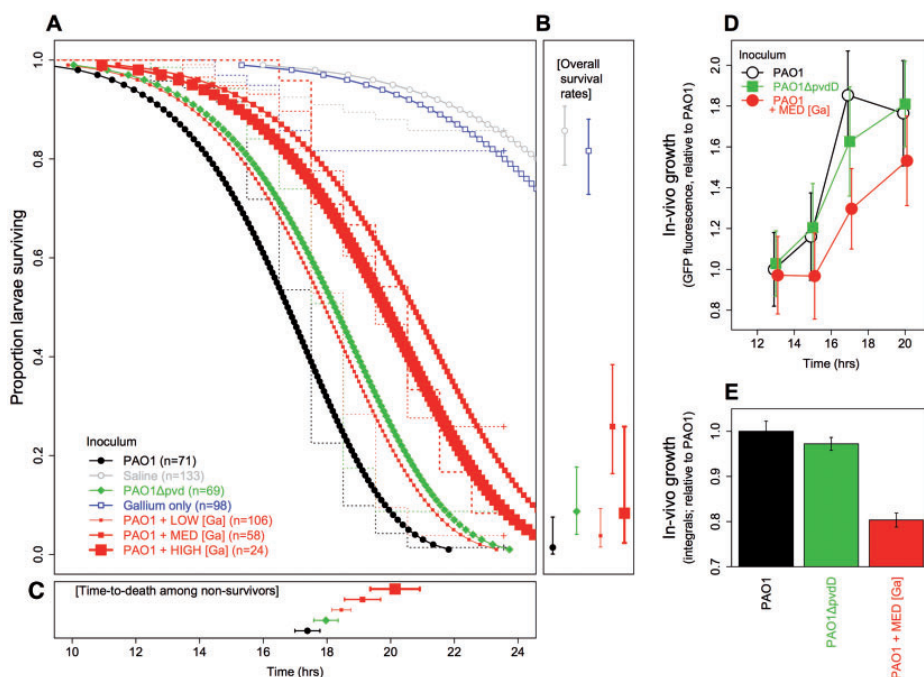
In *in vitro* assays, we found that gallium strongly inhibited bacterial growth, and that the inhibitory effects were mediated primarily via gallium's extracellular quenching activity and not because gallium is toxic *per se* (Fig. 1A). When siderophores were required and could be produced, increasing gallium concentration was associated with a steep decline in growth (slope  $\pm$  SE of regression with  $\log_{10}[\text{Ga}]$ :  $-0.435 \pm 0.011$ ,  $t = -38.04$ ,  $P < 0.001$ ). In contrast, when siderophores were not required and not produced, gallium only weakly affected

growth (slope  $\pm$  SE:  $-0.067 \pm 0.019$ , 95% CI for drop = [2.91–15.86%]; difference in slopes  $0.368 \pm 0.022$ ,  $F_{1,140} = 276.41$ ,  $P < 0.001$ )—particularly over the range of concentrations up to and including 50  $\mu$ M, which correspond to the concentrations likely experienced in our *in vivo* experiments (see below).

It has been suggested that as the benefit of pyoverdine production drops, bacteria should gradually scale back their investment in this trait [19]. On the other hand, it has also been shown that pyoverdine production is upregulated in response to more stringent iron limitation [28], as presumably induced by gallium. Here, we saw a combination of these two regulatory effects, with investment to replace quenched pyoverdine actually increasing from low to intermediate gallium supplementation levels and cessation becoming evident only at higher concentrations (Fig. 1B; ANOVA comparison of quadratic versus linear fits:  $F_{1,36} > 15$ ,  $P < 0.001$  in each case).

Given our *in vitro* observations of gallium's effects on growth and pyoverdine production, we expected it to affect virulence and bacterial fitness *in vivo* too. We tested this in experimental infections of greater waxmoth larvae (*G. mellonella*). Gallium-supplemented *P. aeruginosa* infections indeed showed significantly attenuated virulence compared with non-supplemented infections (Fig. 2A–C; Weibull curve comparison:  $z = 3.10$ – $7.82$ ,  $P < 0.001$  in all cases). Notably, infections supplemented with medium and high concentrations of gallium (corresponding to the intermediate gallium concentration used in the *in vitro* assays, see 'Methodology' section) were significantly less virulent ( $z = 4.96$  and  $2.39$ ,  $P < 0.05$  in both cases) than infections with PAO1 $\Delta$ *pvdD*, a mutant defective for pyoverdine production that itself showed attenuated virulence versus PAO1 ( $z = 3.49$ ,  $P < 0.001$ ). Gallium alone appeared to have little effect on hosts, with levels of virulence not significantly different from those seen in saline-injected controls (Fig. 2A: survival curve comparison:  $z = -0.93$ ,  $P = 0.35$ ; Fig. 2B: pairwise proportion tests for survival rates:  $X_1^2 = 0.43$ ,  $P = 0.51$ ). Bacterial growth *in vivo* was also significantly reduced by gallium (Fig. 2D and E). Growth integrals were lower in gallium-supplemented larvae than in WT-injected larvae (Fig. 2E; Tukey's 95% CIs for the difference: 16.21–21.44%,  $t = 17.24$ ,  $P < 0.001$ ) and, moreover, lower than in larvae injected with the siderophore-defective mutant, PAO1 $\Delta$ *pvdD* (Fig. 2E;

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**Figure 2.** Gallium attenuates *P. aeruginosa* virulence and growth in *G. mellonella* larvae. (A–C) Virulence across treatments, as Kaplan–Meier (stepped lines) and Weibull (smoothed lines) survival curves; proportion surviving (with 95% binomial CIs); and time-to-death (means and 95% CIs). We estimate that inocula with ‘LOW’ (2.5–50  $\mu\text{M}$ ), ‘MED’ (500  $\mu\text{M}$ ) or ‘HIGH’ (2500  $\mu\text{M}$ ) concentrations of  $\text{Ga}(\text{NO}_3)_3$  gave in-host concentrations of  $\sim 0.05$  to  $\sim 50$   $\mu\text{M}$  (see ‘Methodology’ section). (D) Bacterial density *in vivo* (GFP signal in host homogenate; means and 95% CIs from  $\sim 24$  larvae) corrected against saline-injected controls and scaled relative to PAO1 at 13 h. (E) Mean and 95% CIs of bacterial growth integrals derived from bootstrap replicate time series (24 replicate splines) from (D)

Tukey’s 95% CI = 13.58–18.97%,  $t = 14.45$ ,  $P < 0.001$ ).

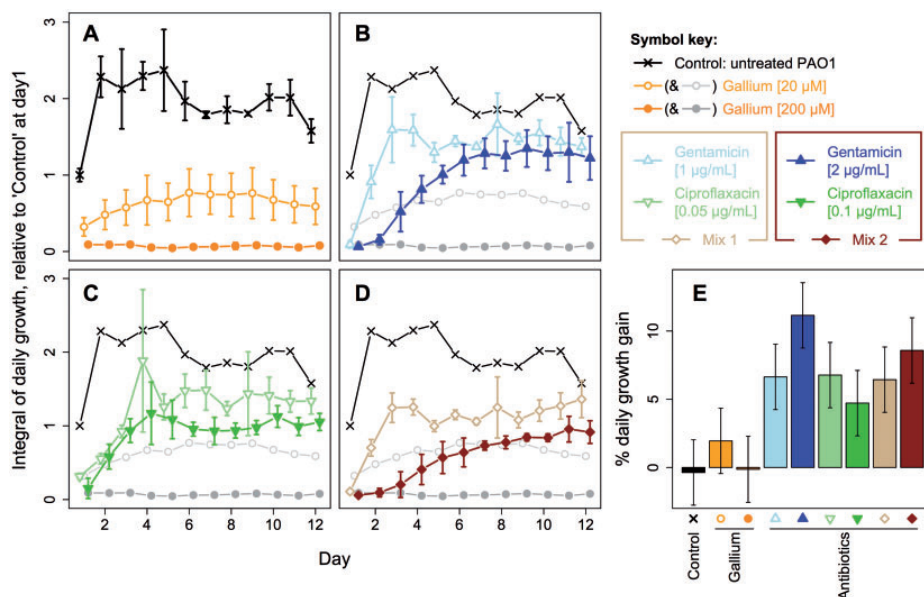
To investigate empirically the general potential for resistance against gallium, we performed experimental evolution with serial batch cultures, comparing *P. aeruginosa* exposed to gallium versus several single- and mixed-antibiotic regimes (Fig. 3A–E). At first, all treatments were strongly refractory to growth, showing 24 h growth integrals no more than a third those of untreated controls (range: 5.8–32.3%). Over the course of a 12-day experiment (a therapy duration that matches clinical standards), however, the growth in all antibiotic treatments increased significantly (Fig. 3E;  $H_0$  slopes = 0: Cp1:  $t = 5.54$ , Cp2:  $t = 3.86$ , Gm1:  $t = 5.43$ , Gm2:  $t = 9.12$ , Mix1:  $t = 5.26$ , Mix2:  $t = 7.02$ ;  $P < 0.001$  in each case), and by the final timepoint their growth integrals were comparable to those of the untreated controls at the start of the experiment (Fig. 3A–D). Gallium-treated cultures, meanwhile, like the untreated control, did not show a significant trend

toward higher growth (Fig. 3E;  $H_0$  slopes = 0:  $t = -0.30$ ,  $P = 0.76$ ;  $t = 1.60$ ,  $P = 0.11$  and  $t = -0.11$ ,  $P = 0.91$  for control, Ga1 and Ga2, respectively).

Per-capita pyoverdine output was generally steady over the course of experimental evolution [Supplementary Fig. S3:  $H_0$  slopes = 0: control:  $z = 0.56$ ,  $P = 0.58$ ; Ga1:  $z = 0.45$ ,  $P = 0.65$ ; all antibiotic treatments pooled (Day 1 excluded):  $z = 0.83$ ,  $P = 0.41$ ], with that of the 20  $\mu\text{M}$  gallium treatment consistently around 2-fold higher than either control or antibiotic-treated cultures (95% CIs for fold-difference were 1.86–2.13 versus control, and 1.96–2.24 versus pooled antibiotic treatments).

In the endpoint phenotypic assays performed under standardized test conditions (CAA and LB media), lines evolved in the Ga1 treatment showed no significant change in pyoverdine production (Fig. 4A) relative to their ancestor (CAA:  $t = 0.81$ ,  $P = 0.43$ ; LB:  $t = 0.08$ ,  $P = 0.94$ ) or to lines evolved under control conditions (CAA:  $t = -0.49$ ,  $P = 0.63$ ; LB:  $t = 0.95$ ,  $P = 0.36$ ), suggesting that the





**Figure 3.** Evolutionary potential for resistance against gallium treatment. (A–D) Over the course of experimental evolution, daily growth integrals for cultures treated with various antibiotics rose significantly, while the growth of gallium treated cultures did not. (E) Slope coefficients for linear fits through data in (A–D), expressed as % of growth of control at Day 1. In all cases, symbols and error bars show means and 95% CIs of six replicate cultures

high pyoverdine output seen during experimental evolution was predominantly a plastic response to gallium (see Fig. 1B). In contrast, the production of pyocyanin did appear to be elevated in the Gal endpoint isolates (Fig. 4B) in CAA medium (versus ancestor:  $t = 3.40$ ,  $P = 0.004$ ; versus control:  $t = 3.09$ ,  $P = 0.008$ ) but not in LB medium (versus ancestor:  $t = 1.69$ ,  $P = 0.12$ ; versus control:  $t = 1.56$ ,  $P = 0.15$ ).

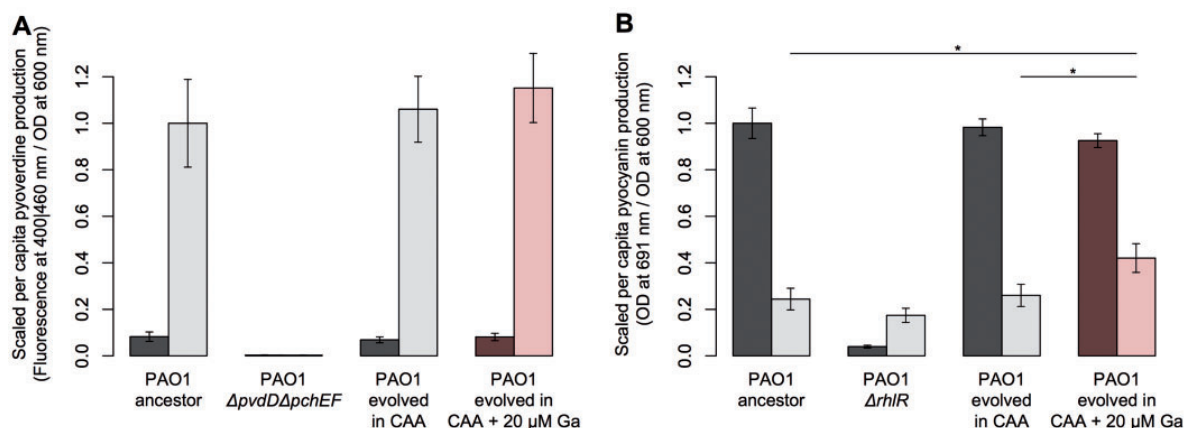
## DISCUSSION

The results reported above indicate that gallium inhibits *P. aeruginosa* growth primarily through extracellular interference with its primary siderophore, pyoverdine (Fig. 1A); that this growth inhibition occurs in an infection context too (Fig. 2D and E), along with a significant reduction in virulence (Fig. 2A–C); and that resistance to gallium treatments does not evolve easily—at least not in comparison to two conventional antibiotics we tested (Fig. 3).

For gallium to be both optimally effective and evolutionarily robust as an antibacterial agent, an appropriately calibrated dose will be key. At lower concentrations, efficacy should initially increase with dose, but at too high concentrations, gallium

may increasingly transit across the cell membrane and begin to interfere directly with iron metabolism, causing general toxicity to bacteria and host cells alike (Fig. 1A; [36]). Here, fitness costs are imposed intracellularly at the individual cell level, and not extracellularly at the level of the collective, which would take us back to a classic antibiotic scenario, with more potential ‘routes to resistance’ and greater potential for steep fitness gradients among individual cells. At sub-toxic levels, meanwhile, where gallium acts primarily through siderophore-quenching, resistance should evolve less readily. Furthermore, we saw that the costs and benefits of siderophore investment itself are also non-linear functions of gallium concentration, owing to the existence of a regulatory ‘trap’. Specifically, intermediate concentrations of gallium induced the highest levels of replacement pyoverdine production in bacteria (Fig. 1B), adding further metabolic stress to increasingly iron-limited cells. Our *in vivo* results, which showed that gallium can suppress virulence to levels beyond those seen in pyoverdine-deficient strains (Fig. 2A–C), are consistent with the interpretation that an appropriate dose of gallium not only restricts bacterial iron uptake but can also impose a costly metabolic burden. Given our understanding of the regulation of pyoverdine production, this

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**Figure 4.** Resistance-related phenotypic changes following experimental evolution under gallium treatment. Pyoverdine (A) and pyocyanin (B) production under standardized test conditions (dark bars = LB medium, light bars = CAA medium) of ancestral PAO1, knock-out strains (i.e. negative controls), control lines (evolved without gallium) and gallium-selected lines. Pyoverdine measures are scaled to that of PAO1 in CAA, whereas pyocyanin is scaled to that of PAO1 in LB. Asterisks indicate cases where Ga-selected lines were significantly different from their ancestor and unexposed control lines. Error bars give 95% CIs of 3–6 replicates

hump-shaped association between pyoverdine investment and gallium is to be expected. Positive feedback occurs when incoming  $Fe^{3+}$ -bound siderophores act via the receptor FpvA and the anti-sigma factor FpvR to activate membrane-bound iron-starvation sigma factor PvdS [37]. High cytoplasmic  $Fe^{2+}$  levels, meanwhile, can generate negative feedback. In this case, the  $Fe^{2+}$  induces Fur (ferric uptake regulator)-mediated repression of *pvdS* [38]. At low gallium concentrations, iron uptake into the cell is steady, so negative feedback keeps pyoverdine production at some intermediate level, while at mid-range gallium concentrations, iron uptake becomes increasingly restricted, leading to steady positive feedback, but weaker negative feedback, and consequently, pyoverdine production increases. Finally, at high concentrations, iron uptake may be so severely restricted that the positive feedback loop fails, and pyoverdine production stalls completely. Exploiting metabolic ‘traps’ such as this could significantly increase the effectiveness of treatments, but requires that the associated regulatory networks should be left intact and functional. This raises another point in favor of extracellular quenching strategies, as opposed to, say, intracellular-mediated deactivation of entire molecular pathways.

To what extent should gallium’s antibacterial activity be evolutionarily robust? In our selection experiment (Figs 3 and 4A), we saw little evidence of adaptation to gallium, although perhaps we can still predict what sort of phenotypic changes could

conceivably confer resistance against gallium-mediated siderophore quenching, and under which conditions such adaptations could spread. Below, we consider several potential evolutionary responses, which are discussed further in Table 1.

First, let’s consider pyoverdine loss-of-function mutants, which are known to arise readily under iron limited conditions [39–41]. In co-infection with siderophore producers, non-producing mutants could act as cheats—no longer investing in the PG yet still benefiting from the investment of nearby ancestors [5]. Even as opportunities to cheat dwindled, such mutants could continue to spread, since, disadvantaged as they would be with respect to autonomous iron acquisition, they would at the same time be freed of the substantial extra metabolic burden of pyoverdine production under gallium regimes (see Fig. 1B). Depending on specific conditions within host tissues, the net fitness of non-producers could be not far off that of pyoverdine producers (Fig. 2E), so the mutants could potentially come to occupy a substantial share of the population. We saw no significant change in mean pyoverdine production in strains evolved under gallium (Fig. 4A), suggesting that cheats did not gain prominence in these cultures. However, certain individual lines (three antibiotic lines and one Ga1 line) went extinct during the course of the experimental evolution, and this extinction was in each case accompanied by a crash in per capita pyoverdine production levels (Supplementary Fig. S3), which would be consistent with a scenario




**Table 1.** How likely is resistance against gallium-mediated pyoverdine quenching?

Mutant phenotype	Why resistant?	Likelihood for mutant to arise	Likelihood for mutant to spread
Pyoverdine production reduced or shut down.	No true resistance, as virulence is only partly restored. However, mutants could avoid being 'trapped' into high pyoverdine production (Fig. 1B), which can be a substantial fitness drain (Fig. 2E).	High Pyoverdine-negative mutants arise readily [39, 41].	Low In mixed cultures, gallium reduces total population density and the effective group size at which pyoverdine can be shared, and these effects both disfavor the mutant [45, 46].
Pyoverdine modified to bind iron with greater specificity.	Iron uptake efficiency, and hence growth, should improve.	Low (a) Pyoverdine has already evolved high iron specificity [20]. Further improvements are unlikely. (b) Ga <sup>3+</sup> and Fe <sup>3+</sup> remain fundamentally very similar in binding behavior.	Low (a) Pyoverdine molecules are shared across the local community [47], so producers of the novel and the ancestral pyoverdine types would benefit similarly.
Regulatory shift from producing pyoverdine to producing pyochelin, a secondary siderophore normally deployed in less iron-limited conditions.	Although pyochelin is generally a less effective siderophore than pyoverdine, this strategy could be advantageous under extreme conditions (e.g. in the presence of gallium).	High Regulatory mechanisms already exist to facilitate facultative switching between siderophore types in response to changing iron stress [48]. Mutations that alter this switch could probably arise easily.	Low (a) Like pyoverdine, pyochelin is also a shared trait, so benefits would go to non-mutants too. (b) Gallium can quench pyochelin too, and so it still inhibits iron uptake [49].
Own pyoverdine production reduced + specialization to use heterologous siderophores from other co-infecting species.	Ceasing pyoverdine production would reduce personal costs, and heterologous siderophores could offer compensatory benefits.	Low Although <i>P. aeruginosa</i> can already take up heterologous siderophores (e.g. enterobactin, desferrioxamine) [50], this route would require co-infection with a bacterium that produces an accessible siderophore.	Low (a) Most siderophores (e.g. desferrioxamine) are still prone to bind gallium [51]. (b) Wild-type <i>P. aeruginosa</i> can also facultatively switch to heterologous siderophore use whenever such siderophores become available [50].
Own pyoverdine production reduced + specialization to take up iron directly from the host.	Ceasing pyoverdine production would reduce personal costs, while iron from other sources could offer compensatory benefits.	High <i>P. aeruginosa</i> already possesses the means to take up iron in various forms [50], including when it is in complex with hosts' iron chelators. A simple switch in a regulatory pathway might be all that is required.	Low (a) Some host iron chelators might also bind gallium (e.g. citrate). (b) Wild-type <i>P. aeruginosa</i> can also facultatively switch to alternative uptake mechanisms when such sources become available [50].
Upregulated production of reducing agents (e.g. pyocyanin), which extracellularly reduce ferric to ferrous iron.	Reducing agents increase availability of the more soluble ferrous form of iron (Fe <sup>2+</sup> ), which can be taken up without the need for siderophores.	High Upregulation of an already existing trait could be achieved easily [30].	Low (a) Increased production of a metabolite would induce extra costs. (b) Like pyoverdine, pyocyanin is also a shared trait, so benefits (in the form of ferrous iron) would go to non-mutants too.

Here, we consider various mutant phenotypes that could putatively confer resistance, and propose hypotheses regarding the likelihood of emergence and spread in each case.

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of siderophore-non-producing cheats spreading in these cultures. In any event, the rise of such mutants should still lead to less virulent infections (Fig. 2A–C; [42–44]).

Alternative scenarios for evolutionary responses to gallium treatment could involve modifying pyoverdine to have substantially greater affinity for  $\text{Fe}^{3+}$  than for  $\text{Ga}^{3+}$ , or switching to ‘backup’ siderophores relatively less susceptible to gallium (Table 1). Such mutations could conceivably arise but in each scenario we would expect attendant selection for the mutation to be relatively weak because, as PGs, these alternative or modified siderophores’ benefits would still be accessible to all cells within diffusion range, including those lacking the novel mutation. In addition, gallium and iron remain fundamentally similar in their physical properties, such that gallium will still bind—to some extent at least—any modified siderophore.

Further possible evolutionary responses could involve mutants that specialize in the direct uptake of  $\text{Fe}^{3+}$ -containing compounds produced by other competing microbes (i.e. inter-specific cheats), or present as chelators in the host tissues. Such mutations are also conceivable, given that bacteria already possess a diversity of iron-uptake machineries [50]. However, considering that gallium can displace  $\text{Fe}^{3+}$  from other compounds too, it is not clear that such strategies would offer any clear advantages over siderophore-mediated uptake.

Finally, bacteria could potentially sidestep their dependence on the  $\text{Fe}^{3+}$  form of iron (prevalent under oxygen replete and neutral pH conditions) by altering their environment to increase the extracellular availability of the more bio-available  $\text{Fe}^{2+}$  ions. Indeed, overproducers of pyocyanin, a redox-active metabolite, have recently been reported to be refractory to gallium [30], and in our own experiments, we did see a weak but significant mean increase in pyocyanin production under certain conditions among cultures evolved under gallium treatment (Fig. 4B). However, such metabolites are themselves PGs, so the spread of over-producers could be constrained in due course by the free-loading behavior of variants that produce less, yet still benefit by the increased availability of  $\text{Fe}^{2+}$  ions.

In our experimental infections, we observed that gallium supplementation reduced both the virulence and the in-host fitness of *P. aeruginosa* (Fig. 2). However, pathogen fitness and virulence will not always be strongly positively correlated [43, 52]. For example, we showed that intermediate gallium

induced overexpression of pyoverdine (Fig. 1B), and in some contexts, this could potentially lead to higher virulence, given that pyoverdine production is linked to certain other virulence factors [53, 54]. Indeed, while gallium is generally known to reduce virulence [19], one recent study [55] showed that in very dense cultures, gallium supplementation actually upregulated production of certain virulence factors. Thus, while gallium represents a promising way to reduce bacterial load, its overall effectiveness in reducing damage to a host will, as always, depend also on the particular characteristics of the host and its interaction with the pathogen.

#### CONCLUSIONS AND IMPLICATIONS

Gallium has seen application in medical contexts for years (e.g. as an anti-cancer drug [56]) and has previously been proposed, and tested, as a treatment against bacterial infections [19, 25, 57, 58]. Gallium can be directly toxic at high concentrations, but here, working with concentrations below this toxic range, we have focused on its capacity to indirectly affect bacteria through disruption of siderophore-mediated iron uptake. Specifically, gallium quenches siderophores extracellularly, starving cells of iron and pushing them into a metabolically costly regulatory trap from which there seems to be little scope for evolutionary escape. In light of our results, we contend that this approach—and more generally the extracellular targeting of PGs—could curb microbial virulence in an evolutionarily robust manner, and therefore represents a promising alternative to our dwindling succession of traditional antibiotics [59–61].

#### SUPPLEMENTARY DATA

Supplementary data are available at *EMPH* online and at the Dryad depository: doi:10.5061/dryad.8kk36.

#### ACKNOWLEDGEMENTS

We thank A.R. Hall, A. Buckling, S.P. Diggle and F. Gorostidi for discussion, and Ramon Weishaupt and Zoé Dumas for help in the laboratory.

#### FUNDING

This work was funded by two grants from the Swiss National Science Foundation (R.K.), a Marie Curie Reintegration grant from the European Commission (R.K.) and the Centre for

Immunity, Infection and Evolution, University of Edinburgh (WT grant 095831, S.P.B.).

**Conflict of interest:** None declared.

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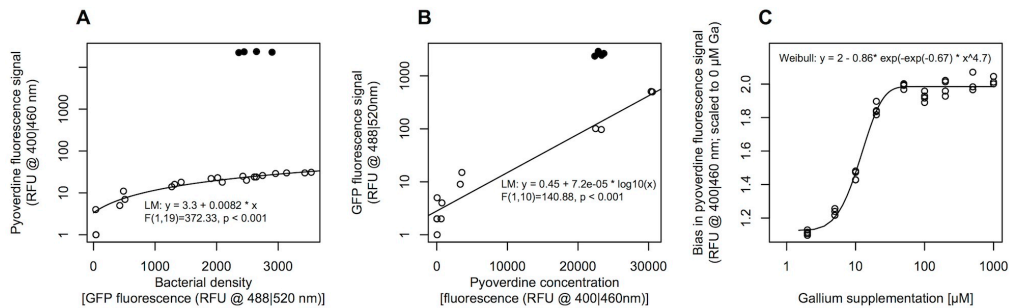
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### 3 Gallium-Mediated Siderophore Quenching as an Evolutionarily Robust Antibacterial Treatment

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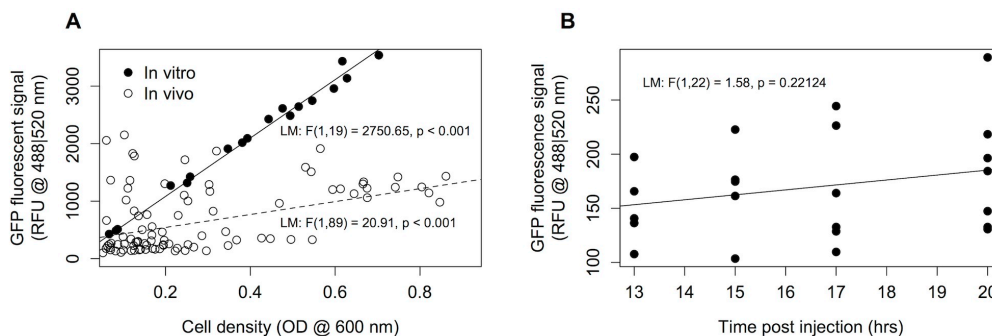
## 3.1 Supporting Material

Figure S1.



**Figure S1.** Reliability of proxy measures for pyoverdine. (A) Differentially diluted 24-hr LB cultures of PAO1 $\Delta$ *pvdD-gfp*, a strain constitutively expressing GFP yet defective for pyoverdine production, still show some signal in the pyoverdine fluorescence channel (400|460 nm) that correlates significantly with their level of GFP fluorescence (488|520 nm; open symbols). (B) Conversely, purified and resuspended pyoverdine, diluted to different concentrations in 0.8% saline, similarly showed some signal leakage into the GFP channel (open symbols). In both cases, however, the magnitude of signal leakage was minor compared to magnitude of signal recorded under the conditions of our primary experiments. To illustrate this, we have overlaid on both panels the same subset of data from Fig. 1, where PAO1*pvdA-gfp* was grown under iron-limited conditions without added gallium, and assayed for both measures (filled symbols). (C) A 200  $\mu$ M dose of purified pyoverdine in our standard iron-limited CAA medium, when supplemented with 50  $\mu$ M or more of gallium, showed a nearly 2-fold higher signal in the 400|460 nm pyoverdine fluorescence channel. Alterations of the fluorescent properties of pyoverdine when bound to iron, unbound, or bound to gallium have previously been described, and where appropriate we applied correction factors derived from the fitted curves shown here to account for this potential bias in our data.

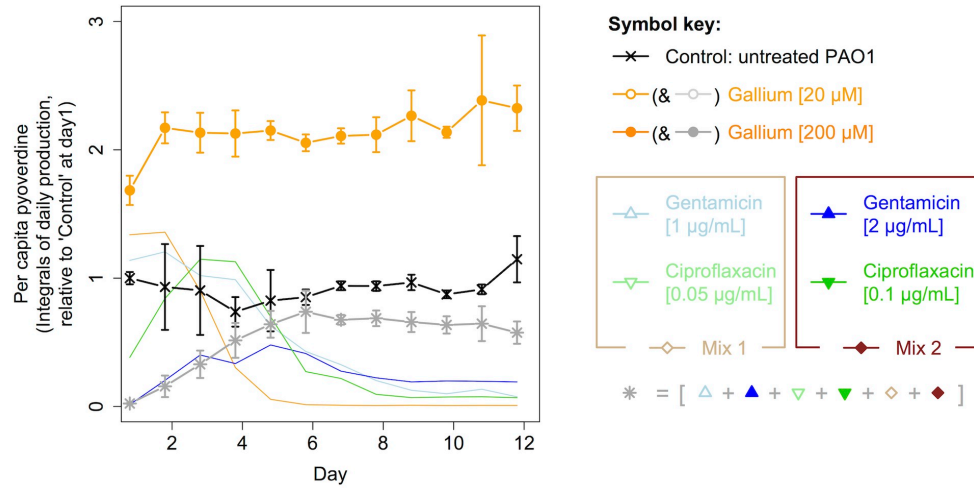
Figure S2.



**Figure S2.** Reliability of GFP-signal as a proxy for growth of bacteria constitutively expressing GFP. (A) GFP-signal produced by PAO1-*gfp* correlates significantly with cell density *in vitro* (measured as optical density at 600 nm of cultures after 24hr growth in iron-limited CAA medium; filled symbols and solid line) and *in vivo* (homogenate recovered from *Galleria mellonella* larvae experimentally infected with PAO1-*gfp* as described in main text, and here pooled across all blocks and time-points sampled; open symbols and dotted line). Summary statistics for simple linear regressions are provided in each case. (B) As expected, GFP-signal does not significantly increase over time in saline-injected larvae and, at the final timepoint, remains  $5.85 \pm 0.72$  SE fold lower than the signal from larvae injected with the poorest performing of bacterial strains (i.e. PAO1*pvdD-gfp*), suggesting that auto-fluorescence from larval tissues was negligible.

### 3.1 Supporting Material

Figure S3.



**Figure S3.** Pyoverdine production during experimental evolution of *P. aeruginosa* under gallium or antibiotic treatments. Per-capita pyoverdine production (400|460nm / OD at 600 nm) was consistently ~2 fold higher in 20 µM gallium-treated cultures than in other treatments. Thin lines depict three individual replicates in which growth crashed, attended by concomitant declines in pyoverdine production. Symbols and error bars show means and 95% CIs of 6 replicate cultures.

## CHAPTER 4

# Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections

This research was originally published in *Evolutionary Applications*

**Weigert, M., Ross-Gillespie A., Leinweber A., Pessi G., Brown S.P., and Kümmerli R.**, 2017, Manipulating virulence factor availability can have complex consequences for infections, *Evolutionary Applications* 10 (1): 91-101.

doi: 10.1111/eva.12431



# Manipulating virulence factor availability can have complex consequences for infections

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## Funding information

The Swiss National Science Foundation, Grant/Award Number: PP00P3-139164; the Novartis Foundation for medical-biological research, Grant/Award Number: 14B0457; DAAD; HFSP, Grant/Award Number: RGP0011/2014

## Abstract

Given the rise of bacterial resistance against antibiotics, we urgently need alternative strategies to fight infections. Some propose we should disarm rather than kill bacteria, through targeted disruption of their virulence factors. It is assumed that this approach (i) induces weak selection for resistance because it should only minimally impact bacterial fitness, and (ii) is specific, only interfering with the virulence factor in question. Given that pathogenicity emerges from complex interactions between pathogens, hosts and their environment, such assumptions may be unrealistic. To address this issue in a test case, we conducted experiments with the opportunistic human pathogen *Pseudomonas aeruginosa*, where we manipulated the availability of a virulence factor, the iron-scavenging pyoverdine, within the insect host *Galleria mellonella*. We observed that pyoverdine availability was not stringently predictive of virulence and affected bacterial fitness in nonlinear ways. We show that this complexity could partly arise because pyoverdine availability affects host responses and alters the expression of regulatorily linked virulence factors. Our results reveal that virulence factor manipulation feeds back on pathogen and host behaviour, which in turn affects virulence. Our findings highlight that realizing effective and evolutionarily robust antivirulence therapies will ultimately require deeper engagement with the intrinsic complexity of host–pathogen systems.

## KEYWORDS

antivirulence therapy, *Galleria mellonella*, host effects, opportunistic pathogen, pathogen fitness, pleiotropy, *Pseudomonas aeruginosa*, siderophore

## 1 | INTRODUCTION

The pervasive idea that virulence—the damage a host experiences during infection—follows more or less directly from pathogen load has shaped our view of infectious disease since the early days of germ theory (Anderson & May, 1979; Bastian, 1875; Evans, 1976; Frank, 1996; Pasteur, 1880; Stearns & Koella, 2008) and has underpinned our clinical quest to eradicate harmful microbes (Allison, Brynildsen, & Collins, 2011; Dagan, Klugman, Craig, & Baquero, 2001; Russell,

2011). However, advances over the years have revealed that the severity of an infectious disease depends on much more than just the sheer number of pathogens present; rather, it derives from complex interactions between the pathogen, its host and the prevailing abiotic and biotic ecological conditions (Bull & Luring, 2014; de Lorenzo, 2015; Méthot & Alizon, 2014; Schmid-Hempel, 2011). In other words, a microbe's pathogenicity is not so much about what it is and how abundant it is, but what it does, when it does it and to whom.

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# 4 Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections

These insights have important consequences for antibacterial therapies that seek to control rather than eradicate infections (Vale et al., 2016). In particular, “antivirulence” approaches have been seen as promising alternatives to classic antibiotics (Allen, Papat, Diggle, & Brown, 2014; Cegelski, Marshall, Eldridge, & Hultgren, 2008; Rasko & Sperandio, 2010; Vale et al., 2016). Such therapies seek to disarm rather than kill pathogens and do so by inhibiting the synthesis or the functioning of virulence factors (e.g. toxins, tissue-degrading enzymes, iron-scavenging siderophores, quorum sensing signals; Rahme et al., 1995; Miethke & Marahiel, 2007; Nadal Jimenez et al., 2012; LaSarre & Federle, 2013). The appeal of this strategy is that any effects on bacterial fitness should be relatively minor, and therefore, such treatments should induce only relatively weak selection for resistance (André & Godelle, 2005; Pepper, 2012). However, given the above-mentioned complexities intrinsic in infectious diseases, we can expect that in many cases, a given antivirulence drug will have effects that extend beyond simply quenching the targeted virulence factor. We might have all sorts of unanticipated secondary effects on the behaviour of the pathogen and its host. For example, the suppression of one virulence factor could pleiotropically affect the regulation of another virulence factor due to regulatory linkage at the genetic level (Balasubramanian, Schnepfer, Kumari, & Mathee, 2013; García-Contreras et al., 2014; Herrera, García-Arriaza, Pariente, Escarmis, & Domingo, 2007; Nadal Jimenez et al., 2012). Furthermore, virulence factors often serve as cues for hosts to mount an immune response (Miyashita, Takahashi, Ishii, Sekimizu, & Kaito, 2015; Park et al., 2014; Schmid-Hempel, 2005; Taszlow & Wojda, 2015), so interfering with some virulence factors' availability could indirectly modulate host responses.

In the light of this inherent complexity, it seems challenging to predict how a specific antivirulence therapy will likely affect bacterial load and treatment efficacy. If indeed the treatment causes secondary effects of the sort envisaged above, we might need to carefully re-evaluate previous claims on the evolutionary robustness of such therapies. Complex interactions between pathogen and host factors could bring into play a multitude of different traits, all of which would be potential targets upon which natural selection could act on. Consequently, there could still be considerable selection for pathogen variants that are resistant to the treatment and/or become more virulent (Vale, Fenton, & Brown, 2014; Vale et al., 2016).

Here, we use the opportunistic human pathogen *Pseudomonas aeruginosa* as a test case to investigate the consequences of manipulating virulence factor availability. This bacterium relies on a number of virulence factors to establish infections in animals and humans, including immune-compromised cystic fibrosis patients (Lyczak, Cannon, & Pier, 2002; Papaioannou, Utari, & Quax, 2013; Rahme et al., 2000). One particularly well-studied virulence factor is pyoverdine, a siderophore secreted into the local environment to scavenge iron from host tissue (Cornelis & Dingemans, 2013; Harrison, Browning, Vos, & Buckling, 2006; Meyer, Neely, Stintzi, Georges, & Holder, 1996). Pyoverdine is a multifunctional molecule. It can be shared as public good between cells for iron uptake to stimulate growth and biofilm formation (Banin et al., 2008; Buckling et al., 2007). It is also used as a

signalling molecule to control its own expression, and the synthesis of two additional virulence factors, exotoxin A and protease IV (Lamont, Beare, Ochsner, Vasil, & Vasil, 2002). Additionally, it can act as a toxin by interfering with mitochondrial iron homeostasis (Kirienko, Ausubel, & Ruvkun, 2015). For all those reasons, pyoverdine has been identified as a suitable target for antivirulence therapies (Banin et al., 2008; Bonchi, Frangipani, Imperi, & Visca, 2015; Bonchi, Imperi, Minandri, Visca, & Frangipani, 2014; Kaneko, Thoendel, Olakanmi, Britigan, & Singh, 2007; Lamont et al., 2002; Ross-Gillespie, Weigert, Brown, & Kümmerli, 2014; Visca, Imperi, & Lamont, 2007). In this study, we manipulated the availability of pyoverdine in the context of experimental infections of greater waxmoth larvae (*Galleria mellonella*). We investigated how interference with this virulence factor affects (i) bacterial growth within the host; (ii) the host's response to infections; (iii) the pleiotropic regulatory links to other virulence factors; and (iv) how these factors combine and determine the overall level of virulence the host experiences. Building from previous work, we reduced the in vivo availability of pyoverdine by supplementing bacterial inocula with gallium, an iron mimic that inactivates pyoverdine molecules by binding irreversibly to them in place of iron (Kaneko et al., 2007; Ross-Gillespie et al., 2014). In addition, we also explored pathogen and host responses under conditions of increased pyoverdine availabilities. This allows us to test more generally how predictive virulence factor availability is for disease severity.

## 2 | MATERIALS AND METHODS

### 2.1 | Strains and media

Our experiments featured the clinical isolate *P. aeruginosa* PAO1 (ATCC 15692), a pyoverdine-defective knockout strain derived from this wild type (PAO1 $\Delta$ pvdD), and three derivatives of these strains engineered via chromosomal insertion (*attTn7::ptac-gfp*, *attTn7::ptac-mcherry*) to constitutively express fluorescent proteins—that is PAO1-*gfp*, PAO1-*mcherry* and PAO1 $\Delta$ pvdD-*gfp*. Overnight cultures were grown in 8 ml Luria-Bertani (LB) medium in 50-ml Falcon tubes and incubated at 37°C, 200 rpm for 16–18 hr. For all experiments, we subsequently diluted the overnight cultures in 0.8% NaCl saline solution. For in vitro assays, we used iron-limited CAA medium (per litre: 5 g casamino acids, 1.18 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.25 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 µg/ml human apotransferrin, 20 mM NaHCO<sub>3</sub> and 25 mM HEPES buffer). Human apotransferrin in combination with NaHCO<sub>3</sub> (as cofactor) is a strong iron chelator, which prevents non-siderophore-mediated iron uptake. All chemicals were purchased from Sigma-Aldrich, Switzerland. Pyoverdine was isolated using the protocol by Meyer et al. (1997).

### 2.2 | Manipulation of pyoverdine availability

In both our in vitro and in vivo assays, we reduced and increased pyoverdine availability by supplementing bacterial inocula with, respectively, either gallium nitrate or purified pyoverdine. Gallium is an iron mimic that inactivates pyoverdine molecules by binding irreversibly

to them in place of iron. It thereby lowers pyoverdine availability in a dose-dependent manner (Kaneko et al., 2007; Ross-Gillespie et al., 2014). The addition of pyoverdine immediately increases availability after inoculation, which has been shown to stimulate bacterial growth *in vitro* (Kümmerli & Brown, 2010). For *in vitro* experiments, we varied gallium and pyoverdine concentrations from 5 to 250  $\mu\text{M}$ . For *in vivo* experiments, we prepared inocula with 10-fold higher concentrations, as we assumed that upon injection into a host larva's haemolymph (a total volume of approximately 100  $\mu\text{l}$ ; Harding, Schroeder, Collins, & Frankel, 2013), our infection inoculum (a 10  $\mu\text{l}$  volume) would become diluted by a factor of approximately ten. Hereafter we report *in vivo* concentrations as estimated final concentrations, adjusted to reflect this assumed 10-fold dilution.

### 2.3 | *In vitro* growth and pyoverdine assays

To assess how our treatment regimes affect pyoverdine availability and bacterial growth, we performed *in vitro* growth assays. Overnight LB cultures (PAO1 and PAO1 $\Delta\text{pvdD}$ ) were washed twice and standardized for optical density ( $\text{OD} = 2$ ) and then inoculated at  $10^{-3}$  dilution to iron-limited CAA supplemented with either gallium nitrate ( $\text{Ga}(\text{NO}_3)_3$ ; 5, 10, 20, 50 and 250  $\mu\text{M}$ ) or purified pyoverdine (same concentrations), to respectively reduce or enhance the availability of pyoverdine. All conditions were carried out in fourfold replication. Growth was tracked over 24 hr (37°C) in 200  $\mu\text{l}$  cultures in 96-well plates (BD Falcon, Switzerland) using a Tecan Infinite M-200 plate reader (Tecan Group Ltd, Switzerland). We measured OD at 600 nm and pyoverdine-associated fluorescence (400 ex | 460 em), every 15 min following brief shaking of the plate (30s, 3.5 mm orbital displacement). As gallium increases pyoverdine fluorescence, we corrected fluorescence values using a previously published calibration curve (Ross-Gillespie et al., 2014).

### 2.4 | *In vivo* growth assays

Infections were performed following protocols described in Ross-Gillespie et al. (2014). Briefly, final-instar *Galleria mellonella* larvae, standardized for mass and general condition, were surface-sterilized with 70% ethanol, inoculated between the posterior prolegs (Hamilton syringe; 26s gauge sterile needle) and then individually (randomly) distributed to the wells of 24-well plate for incubation at 37°C. *In vivo* bacterial growth was assayed as per Ross-Gillespie et al. (2014), using GFP fluorescence signal as a proxy for growth. For this reason, we infected larvae with bacterial strains harbouring a constitutively expressed *gfp* marker (i.e. PAO1-*gfp* or PAO1 $\Delta\text{pvdD}$ -*gfp*). Inocula (10  $\mu\text{l}$ ) contained ~25 colony-forming units (CFU) of either PAO1-*gfp* supplemented with gallium (50  $\mu\text{M}$  or 250  $\mu\text{M}$ ) or pyoverdine (50  $\mu\text{M}$  or 250  $\mu\text{M}$ ), no pyoverdine or the pyoverdine-defective PAO1 $\Delta\text{pvdD}$ -*gfp* as a control treatment. A growth-negative control included the injection of saline solution. At 17 hr postinfection, larvae (24 per treatment) were processed to estimate their bacterial load. Approximately 50% of the larvae that had been infected with the wild-type strain were already dead at this time point. Larvae were individually flash-frozen in

liquid nitrogen and then ground to fine powder using sterile micropes- tles. Powderized larval homogenates were resuspended in 1 ml sterile  $\text{H}_2\text{O}$  and centrifuged at 6300 RCF for 2 min. Thereafter, 200  $\mu\text{l}$  of the water-soluble liquid phase of each sample was transferred to a 96-well plate and assayed for GFP-associated fluorescence using a Tecan Infinite M-200 plate reader. To examine whether the bacterial load at 17 hr postinfection is representative of within-host growth dynam- ics, we repeated the experiment for a subset of treatments (untreated wild type, wild type with intermediate (50  $\mu\text{M}$ ) gallium or pyoverdine concentration, pyoverdine-deficient mutant, saline control). At 13, 15, 17 and 20 hr, we processed randomly selected larvae (24 per treat- ment) as described above and measured their bacterial load.

### 2.5 | *Ex vivo* growth assays

We investigated the potential influence of host effects on bacterial dynamics via *ex vivo* growth assays in haemolymph. In a first step, we primed *G. mellonella* larvae by inoculating them with bacterial wild- type cultures featuring manipulated levels of pyoverdine (by supple- menting inoculum with either intermediate (50  $\mu\text{M}$ ) concentrations of gallium or pyoverdine). As controls, we primed larvae by infecting them with either the pyoverdine-deficient strain, pyoverdine alone, heat-killed wild-type bacteria or the saline control. In a second step, we then measured bacterial growth in haemolymph extracted from these primed larvae. The priming inocula were administered as per the infection protocol described above. Inoculated larvae were dis- tributed, in groups of 4, to petri dishes and incubated at 37°C. After 14 hr, the petri dishes were placed on ice for 15 min to anaesthetize the larvae prior to haemolymph extraction. A small incision was made in the posterior segment using a sterile scalpel, and haemolymph was drained with the aid of gentle pressure (Harding et al., 2013). From each sample, 25  $\mu\text{l}$  of haemolymph was immediately stabilized with 15  $\mu\text{l}$  of an ice-cold pH 6.5 cacodylate buffer (10 mM  $\text{Na-C}_2\text{H}_7\text{AsO}_2$  and 5 mM  $\text{CaCl}_2$ ) and 15  $\mu\text{l}$  of a saturated propylthiouracil solution to inhibit melanization. Samples were then centrifuged (514 RCF, 2 min) to separate the liquid haemolymph fraction from any solid tissue con- taminants, and 30- $\mu\text{l}$  aliquots were transferred to individual wells of a 96-well plate, each containing 70  $\mu\text{l}$  of saline solution. To kill the prim- ing strains and any other bacteria that may have been present in the haemolymph as part of the natural larval microbiota, we added gen- tamicin to the haemolymph/buffer mixture to a final concentration of 20  $\mu\text{g}/\text{ml}$  (a concentration known to kill susceptible *P. aeruginosa*; Choi et al., 2005). Subsequently, we inoculated wells with bacteria from an overnight culture (adjusted to an  $\text{OD} = 2$  and subsequently diluted to  $10^{-4}$ ) of a gentamicin-resistant PAO1-*mCherry* strain (this strain showed the same growth pattern as the untagged wild-type strain). The plate was transferred to a Tecan Infinite M-200 plate reader for 24 hr of incubation at 37°C. Every 15 min, we measured cell density (measured via the *mCherry*-associated fluorescence: 582 ex | 620 em; note: using optical density as a proxy for cell density is not reliable in this naturally turbid medium). These experiments allowed us to ascer- tain (i) whether bacterial growth in haemolymph is affected by a host's history of prior infection and (ii) whether the availability of pyoverdine

during priming predicts subsequent bacterial growth. Note that residual pyoverdine from the priming inocula was below detection limit after haemolymph extraction, and therefore should not influence later bacterial growth patterns.

## 2.6 | Molecular investigation of pyoverdine-mediated pleiotropy

Because pyoverdine is not only a virulence factor but also a signalling molecule, manipulating pyoverdine availability might also affect, via interaction with the PvdS iron-starvation sigma factor, the production of two additional virulence factors, exotoxin A and protease IV (Lamont et al., 2002; Ochsner, Johnson, Lamont, Cunliffe, & Vasil, 1996; Wilderman et al., 2001). We used qPCR to explore whether our extrinsic manipulation of pyoverdine levels could change pyoverdine-mediated signalling and therefore pleiotropically affect expression of genes for virulence factor production (*pvdS*, *toxA* coding for exotoxin A, *prpL* (alternative name: *piv*) coding for protease IV and *pvdA* coding for one of the pyoverdine synthesis enzymes). PAO1 cells were grown until early- and mid-exponential growth phases in 20 ml standard CAA (in a sterile 500-ml Erlenmeyer) containing either (i) no supplement, (ii) 10  $\mu\text{M}$   $\text{Ga}(\text{NO}_3)_3$  (to reduce pyoverdine availability), (iii) 200  $\mu\text{M}$  purified pyoverdine (to increase pyoverdine availability) or (iv) 100  $\mu\text{M}$   $\text{FeSO}_4$  (our negative control under which pyoverdine production should be completely switched off; Kümmerli, Jiricny, Clarke, West, & Griffin, 2009). RNA was extracted using a modified hot acid phenol protocol and purified as in Pessi et al. (2007, 2013). Residual DNA in the sample was eliminated using RQ1 RNase-free DNase I, and purification was performed using the RNeasy Mini Kit (Qiagen). Absence of DNA was verified by PCR using the primers specified in Table S1, and 40 cycles with the GoTaq Polymerase (Promega, Switzerland). RNA quality in the purified samples was then assessed using RNA Nano Chips (Agilent 2100 Bioanalyzer; RIN (RNA integrity number) >7.6). First-strand cDNA synthesis with 10  $\mu\text{g}$  of total RNA from each sample was performed with M-MLV reverse transcriptase RNase H Minus (Promega) and random primers (Promega). cDNA was subsequently purified with the MinElute PCR Purification Kit (Qiagen). The expression of *Pseudomonas aeruginosa* PAO1 genes PA2399 (*pvdD*), PA2426 (*pvdS*), PA1148 (*toxA*) and PA4175 (*prpL*) was analysed with a Stratagene MX300P instrument (Agilent) using GoTaq qPCR Master Mix (Promega). All PCRs were analysed in triplicate with three cDNA dilutions (15, 7.5 and 3.25 ng) and 0.4  $\mu\text{M}$  of individual primers in a total volume of 25  $\mu\text{l}$  per reaction. Primers (Table S1) were designed using Primer3Plus (Untergasser et al., 2007) and subsequently verified by PCR. Fold changes were calculated using the  $\Delta\Delta\text{CT}$  method (Pfaffl, 2001) using the primary sigma factor *rpoD* (PA0576) as housekeeping gene for data normalization.

## 2.7 | Virulence assays

Infections were performed as described above. Inocula (10  $\mu\text{l}$ ) contained  $\sim 25$  CFU of *P. aeruginosa* from an overnight culture,

resuspended in saline solution and supplemented with either gallium nitrate (5  $\mu\text{M}$ , 50  $\mu\text{M}$ , 250  $\mu\text{M}$ ), pyoverdine (10  $\mu\text{M}$ , 50  $\mu\text{M}$ , 250  $\mu\text{M}$ ) or neither. Controls included saline-only, gallium-only (50  $\mu\text{M}$  and 250  $\mu\text{M}$ ) and pyoverdine-only inocula (50  $\mu\text{M}$  and 250  $\mu\text{M}$ ), and also the PAO1 $\Delta pvdD$  strain, defective for pyoverdine production. The vitality of all larvae (i.e. spontaneous movement/response to tactile stimulation) was assessed hourly, starting at 10 hr postinjection. Some of the larvae ( $n = 25$ , 3.14%) either started pupating while under observation or died prematurely during the first 10 hr postinjection—presumably as a result of handling—and hence were excluded from further analyses.

## 2.8 | Statistical analysis

We used the functions from the “grofit” R package to fit spline curves to the growth and pyoverdine production trajectories. From these fitted curves, we extracted growth parameters. In particular, we focused on growth integrals (areas under curves), which combine information from the lag phase, growth rate and yield. Growth integrals are particularly useful for nonlogistic growth trajectories as observed throughout our experiments.

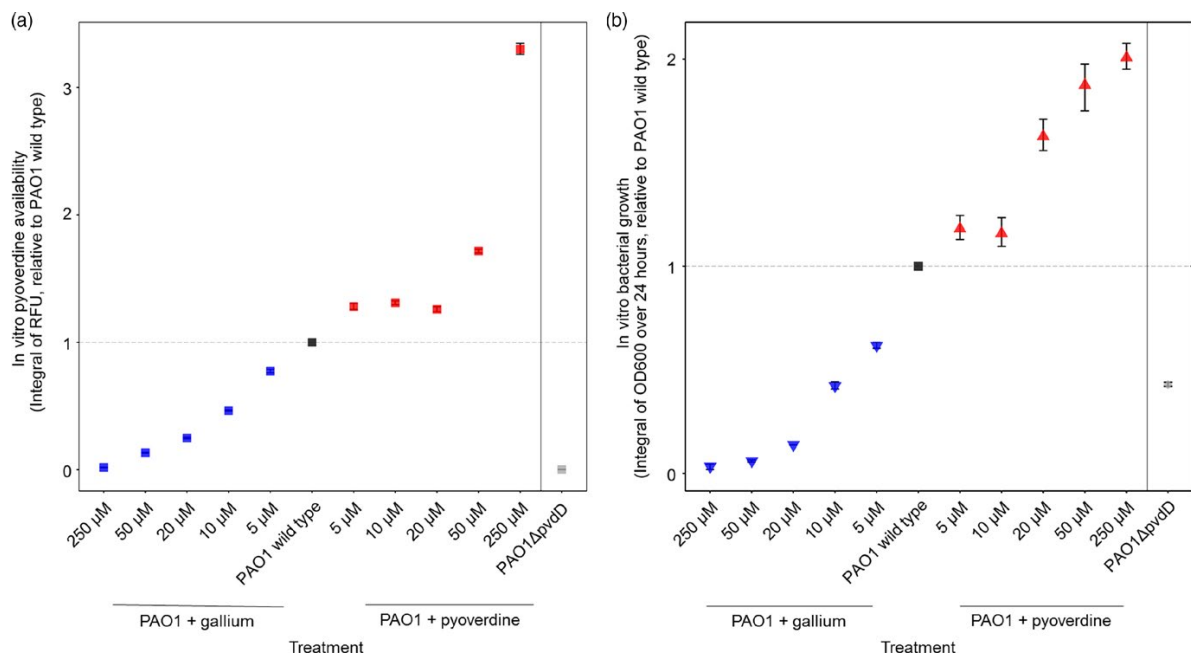
Survival curves were analysed by fitting parametric Weibull survival curves with the aid of functions from the “survival” R package (Therneau & Grambsch, 2000). From the fitted models, we extracted the hazard ratios and used those values to estimate the mortality risk of larvae within each treatment. To confirm the robustness of our analysis, we also performed Cox proportional hazards regression, which yielded qualitatively similar results.

We used both parametric and nonparametric statistical models to test for treatment effects. Specifically, we used Kendall rank correlation analyses to test for associations between pyoverdine availability, growth, host response and virulence. The data from our in vivo and ex vivo growth experiments did not meet the criteria of normally distributed residuals and the homogeneity of variances, which precluded the use of parametric statistical tests. For these analyses, we used the nonparametric Kruskal–Wallis test. All analyses were performed in R 3.3.0 (R Development Core Team 2015).

## 3 | RESULTS

### 3.1 | Treatment effects on in vitro bacterial pyoverdine availability and growth

We first tested whether our treatment regime (i.e. adding gallium to quench pyoverdine or supplementing additional pyoverdine) indeed altered pyoverdine availability as intended. We found that our treatment regime had a positive linear effect on pyoverdine availability (Figure 1a; Kendall's correlation coefficient:  $\tau = .75$ ,  $p < .001$ , measured during the first 8 hr of the growth period when pyoverdine is most needed to overcome iron limitation; Kümmerli & Brown, 2010). Moreover, we found that our manipulation of pyoverdine availability had a significant linear effect on bacterial growth (Figure 1b;  $\tau = .93$ ,  $p < .001$ ): adding gallium reduced growth, while pyoverdine



**FIGURE 1** Our treatment regime significantly affected pyoverdine availability (a) and bacterial growth (b) in linear ways. To capture the dynamics of pyoverdine availability (first 8 hr) and growth (24 hr) in bacterial cultures, we used integrals (i.e. area under the curve) for analysis. Nonlinear patterns in (a), for example for the 10 µM and 20 µM pyoverdine supplementations, can arise because bacteria plastically adjust their pyoverdine production level according to their need (Kümmerli et al., 2009), such that de novo production and supplementation can balance each other out over time. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively, across four independent replicates

supplementation accelerated growth relative to the unsupplemented wild type. Taken together, our in vitro experiments show that our treatment scheme successfully manipulates pyoverdine availability and that pyoverdine is a growth promoter, essential for bacteria to thrive in iron-limited medium.

### 3.2 | Nonlinear effects of pyoverdine availability on in vivo bacterial growth

Pyoverdine availability also had significant effects on bacterial growth within the *G. mellonella* larvae (Kruskal–Wallis test for differences between treatments:  $\chi^2 = 34.80$ ,  $p < .001$ ; Figure 2), but the overall effect was not linear. Instead, bacterial load peaked in infections with the unsupplemented wild type (i.e. at intermediate pyoverdine availability). Both the addition of gallium and pyoverdine significantly reduced bacterial growth compared to unsupplemented wild-type infections (for gallium 50 and 250 µM combined:  $\chi^2 = 8.68$ ,  $p = .013$ ; for pyoverdine 50 and 250 µM combined:  $\chi^2 = 6.66$ ,  $p < .010$ ). Bacterial growth also significantly peaked in infections with the unsupplemented wild type when considering the entire growth trajectories and not only a single time point (Fig. S1), thereby confirming the above pattern.

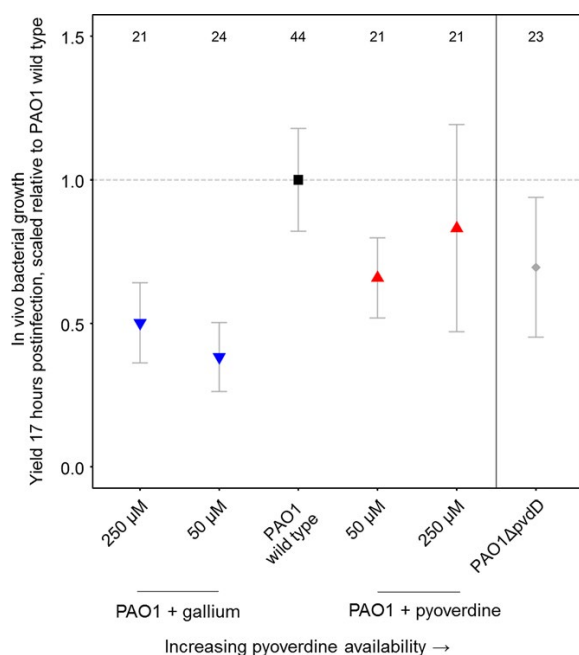
One possible explanation for the absence of a linear relationship between pyoverdine availability and in vivo growth is that pyoverdine might not be required for bacteria to thrive within the host. However,

two control experiments speak against this hypothesis. First, the growth of a pyoverdine-deficient knockout strains was significantly impaired in host infections compared to the wild type (Kruskal–Wallis test:  $\chi^2 = 7.54$ ,  $p < .001$ ; Figure 2). Second, ex vivo growth of wild-type bacteria in extracted haemolymph demonstrated significant iron limitation and high pyoverdine production in this medium (Fig. S2). Altogether, these results indicate that pyoverdine is important for iron scavenging and growth within the larvae.

### 3.3 | Pyoverdine availability affects host responses

To investigate whether bacteria and/or pyoverdine availability triggers variation in host responses, we tracked growth of a wild-type strain ex vivo in haemolymph extracts from larvae previously primed under different conditions. Ex vivo bacterial growth in haemolymph indeed significantly differed depending on the infection history of the larvae (Figure 3; Kruskal–Wallis test:  $\chi^2 = 10.59$ ,  $p = .014$ , including the pyoverdine manipulation regimes and the saline control). Specifically, bacteria showed significantly lower growth in haemolymph from wild-type-primed larvae than in haemolymph from saline-primed larvae ( $\chi^2 = 4.11$ ,  $p = .043$ ). Furthermore, we found a significant negative association between the availability of pyoverdine in the priming inocula and the subsequent ex vivo bacterial growth (Figure 3; Kendall's  $\tau = -.21$ ,  $p = .023$ ). Control experiments revealed that a significant host response can be triggered by multiple stimuli: priming larvae

## 4 Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections

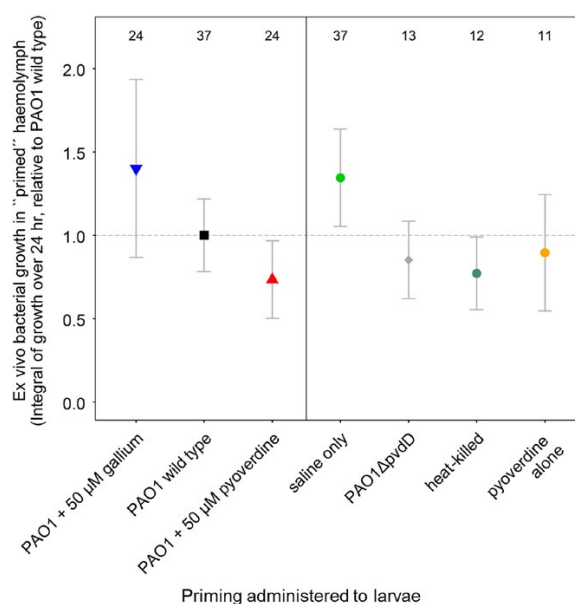


**FIGURE 2** Pyoverdine availability has nonlinear effects on *P. aeruginosa* growth in *G. mellonella* larvae. Bacterial load (measured 17 hr postinfection) peaked in infections with the unsupplemented wild type, while the supplementation of both gallium (blue) and pyoverdine (red) resulted in a significant drop of bacterial load. Infections with a pyoverdine-deficient strain also resulted in a significant growth reduction compared to wild-type infections, indicating that pyoverdine is important for growth in this host. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

with non-pyoverdine-producing bacteria, pyoverdine alone or heat-killed bacteria all resulted in a similarly increased response relative to the saline priming (Kruskal-Wallis test comparing pooled control treatments versus the saline treatment:  $\chi^2 = 8.24$ ,  $p = .004$ ). Overall, our findings suggest that haemolymph primed with bacteria has a growth-inhibiting effect on *P. aeruginosa* and that this effect can vary plastically over time in response to pyoverdine availability.

### 3.4 | Pyoverdine availability affects the expression of other virulence factors

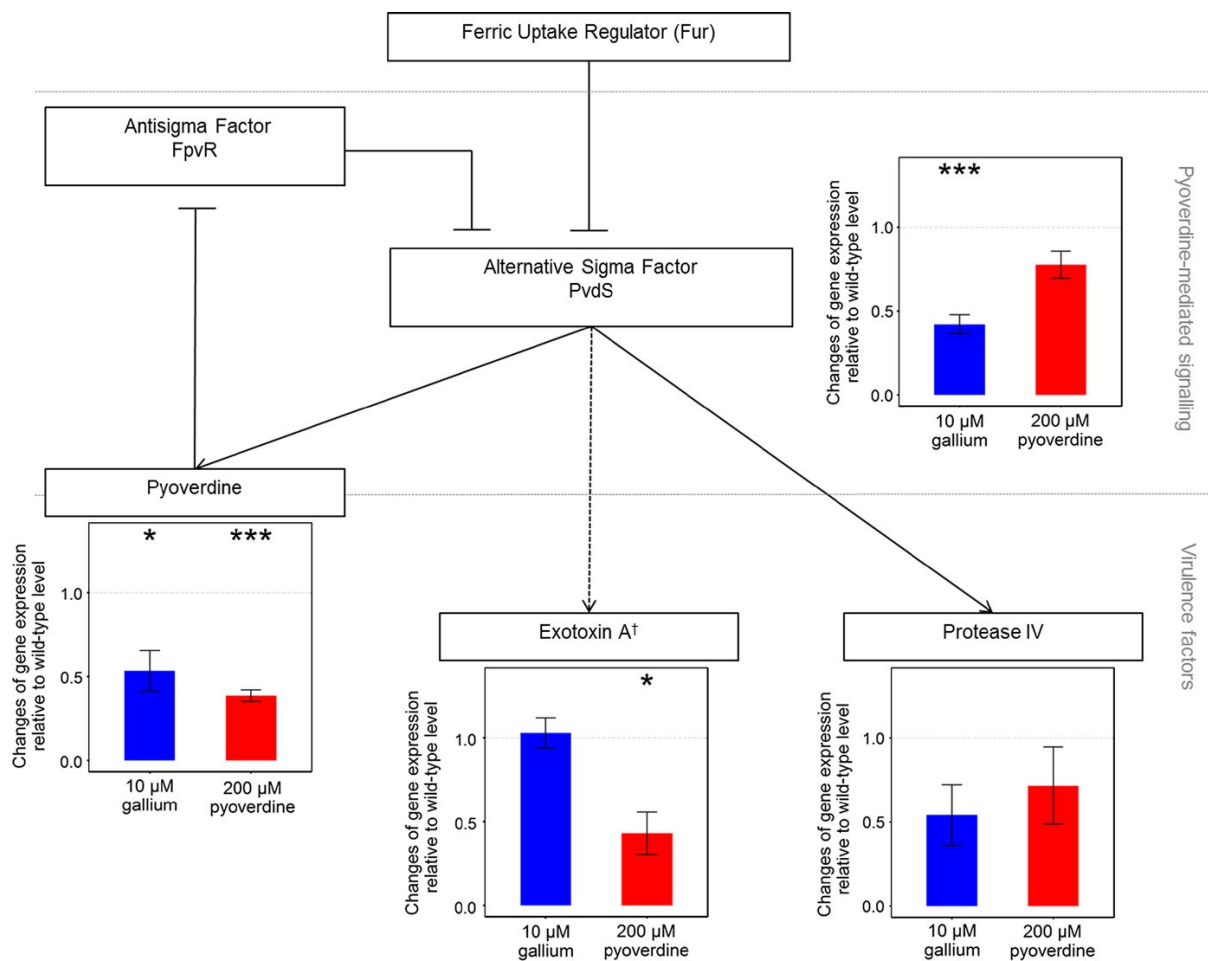
In addition to its function as a siderophore, pyoverdine is also a signalling molecule, which controls its own production and the synthesis of two other virulence factors, namely protease IV and exotoxin A (Beare, For, Martin, & Lamont, 2002; Lamont et al., 2002) (Figure 4). It is therefore well conceivable that the experimental manipulation of pyoverdine availability also affects the expression of these other virulence factors. To test this hypothesis, we performed in vitro qPCR experiments, following the expression of the genes *pvdS*, *pvdA*, *prpL* and *tox A* across three levels of pyoverdine availabilities and two time points (early- and mid-exponential phase). We



**FIGURE 3** Growth of *P. aeruginosa* in haemolymph extracted from primed larvae demonstrates context-dependent host effects. Fourteen hours prior to haemolymph extraction, larvae were primed with wild-type bacteria, either alone or supplemented with gallium or pyoverdine. Control larvae were primed with saline alone, pyoverdine alone, heat-killed wild-type bacteria or a pyoverdine-deficient strain. Haemolymph extracts were gentamicin-treated to kill previously inoculated bacteria, and growth assays were then performed with a gentamicin-resistant wild-type strain. Compared to the saline control, haemolymph primed with wild-type bacteria was significantly more refractory to subsequent bacterial growth, demonstrating a host response to infection. Moreover, we found a significant negative correlation between pyoverdine availability during the priming phase and the subsequent bacterial growth, indicating that pyoverdine is involved in triggering host responses. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

examined these time points because pleiotropy relatively early in the growth cycle is likely to have the biggest effect on subsequent pathogen growth and virulence. The four genes code for the sigma factor PvdS (the main regulator of all three virulence factors), PvdA (enzyme involved in pyoverdine synthesis), protease IV and exotoxin A (Figure 4). Taking the unsupplemented wild-type bacteria growing in our standard iron-limited medium as a reference, we found that the addition of iron dramatically downregulated the expression of all four genes (Table 1). This suggests that all three virulence factors (pyoverdine, protease IV and exotoxin A) are significantly expressed under the imposed iron-limited conditions (see also Ochsner, Wilderman, Vasil, & Vasil, 2002). Next, we examined whether gene expression levels change as a function of pyoverdine availability. We found that pyoverdine manipulation either did not affect gene expression or resulted in the downregulation of interlinked genes (Table 1). As there were no marked differences in gene expression profiles between the early- and the mid-exponential growth phase,





**FIGURE 4** Manipulating pyoverdine availability has moderate effects on the pyoverdine signalling network, and generally leads to the downregulation of interlinked genes. Pyoverdine production is controlled by the alternative sigma factor PvdS that is itself negatively regulated by the ferric uptake regulator FUR, in response to intracellular iron levels. Pyoverdine modulates PvdS activity through a signalling cascade. Incoming iron-bound pyoverdine binds to its cognate receptor, thereby triggering the lysis of the membrane-bound antisigma factor FpvR, which binds and inhibits PvdS. In turn, membrane-released PvdS triggers increased transcription of pyoverdine synthesis genes, but also activates the expression of *prpL* (encoding the protease IV virulence factor) and *toxR* (coding for the ToxR regulator that then stimulates the expression of exotoxin A). Reduced pyoverdine availability (gallium supplementation, blue bars) moderately but significantly reduced *pvdS* and *pvdA* expression. Increasing pyoverdine availability (red bars) moderately but significantly reduced *pvdA* and *toxA* expression. Shown are mean values and standard errors across four replicates. Asterisks indicate significant gene expression changes relative to the unsupplemented wild type ( $p < .05$ ). † indirect regulation via *toxR* regulator

we pooled the data to identify the genes that were significantly downregulated (Figure 4). These analyses revealed that the addition of gallium (10  $\mu\text{M}$ ) slightly but significantly reduced the expression of *pvdS* ( $t_3 = -10.55$ ,  $p = .002$ ) and *pvdA* ( $t_3 = -3.87$ ,  $p = .031$ ). The supplementation of pyoverdine (200  $\mu\text{M}$ ) significantly reduced the expression of *pvdA* ( $t_3 = -17.95$ ,  $p < .001$ ) and *toxA* ( $t_3 = -4.50$ ,  $p = .020$ ). Our results are promising from a therapeutic perspective, as they suggest that the manipulation of pyoverdine availability does not increase the expression of the interlinked virulence factors protease IV and exotoxin A, but rather has a neutral or even a negative effect on their expression.

### 3.5 | Relationship between pyoverdine availability and virulence

Our results presented above (Figures 2–4) show that the manipulation of pyoverdine availability has nonlinear effects on bacterial load, triggers differential host responses and has slight pleiotropic effects on the expression of other virulence factors. How do these factors now all combine within the host and determine the overall level of virulence associated with pyoverdine manipulation? Overall, our experimental infections of *G. mellonella* larvae revealed a significant positive association between pyoverdine availability and virulence

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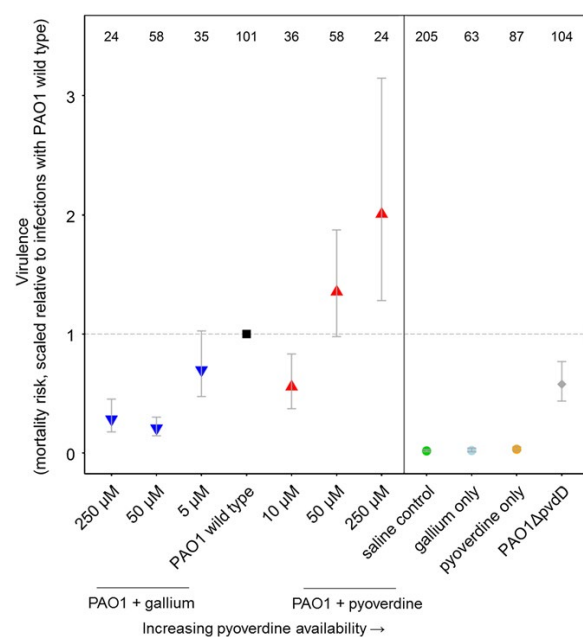
**TABLE 1** Expression fold changes for *P. aeruginosa* genes involved in pyoverdine-mediated signalling

Growth Phase	Gene	Supplementation regime					
		100 $\mu\text{M}$ FeSO <sub>4</sub>		10 $\mu\text{M}$ Ga(NO <sub>3</sub> ) <sub>3</sub>		200 $\mu\text{M}$ Pyoverdine	
		Mean	SE	Mean	SE	Mean	SE
Early-exponential	<i>pvdS</i>	0.0003	0.0002	0.3256	0.0199	0.7237	0.1719
	<i>pvdA</i>	0.0001	0.0000	0.3442	0.0353	0.3675	0.0159
	<i>toxA</i>	0.0544	0.0098	0.9063	0.0161	0.2154	0.0200
	<i>prpL</i>	0.2321	0.0375	0.7072	0.3661	1.1106	0.0821
Mid-exponential	<i>pvdS</i>	0.0003	0.0002	0.5167	0.0350	0.8273	0.0628
	<i>pvdA</i>	0.0003	0.0002	0.7242	0.1163	0.4059	0.0775
	<i>toxA</i>	0.0561	0.0092	1.1514	0.1412	0.6463	0.0519
	<i>prpL</i>	0.0214	0.0046	0.3776	0.0896	0.3240	0.0143

Expression fold changes of *pvdS* (encoding the iron-starvation sigma factor PvdS), *pvdA* (coding for one of the pyoverdine synthesis enzymes), *toxA* (coding for exotoxin A) and *prpL* (encoding protease IV) are expressed relative to the unsupplemented PAO1 wild-type regime.

(Figure 5; Kendall's  $\tau = .71$ ,  $p = .030$ ). Larvae died earlier in infections supplemented with pyoverdine, but survived longer when gallium was added instead. However, the trend was not altogether monotonic: moderately increased pyoverdine availability (10  $\mu\text{M}$ ) significantly decreased rather than increased the virulence risk (parametric survival regression assuming Weibull distribution: coefficient =  $0.082 \pm 0.028$ ,

mean  $\pm$  SE,  $z = 2.89$ ,  $p = .004$ ). Such low-pyoverdine-supplementation infections showed virulence levels comparable to those of unsupplemented infections involving the pyoverdine-deficient PAO1 $\Delta$ *pvdD* mutant (coefficient =  $0.0055 \pm 0.028$ ,  $z = 1.94$ ,  $p = .846$ ). The gallium-supplemented treatments were not ordered monotonically with respect to virulence, in that high (250  $\mu\text{M}$ )-gallium-supplemented infections were no less virulent than intermediate (50  $\mu\text{M}$ )-gallium-supplemented infections (coefficient =  $0.043 \pm 0.037$ ,  $z = 1.18$ ,  $p = .238$ ).



**FIGURE 5** Relationship between pyoverdine availability and virulence, measured as mortality risk of larvae within each treatment. Overall, there is a positive correlation between pyoverdine availability and virulence with a notable exception. Supplementing the infection with 10  $\mu\text{M}$  pyoverdine reduced virulence comparable to a pyoverdine-deficient mutant. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

## 4 | DISCUSSION

Our results show that the manipulation of pyoverdine, an important virulence factor of the opportunistic human pathogen *P. aeruginosa*, affects bacterial load in infections of *G. mellonella* larvae in complex ways, triggers differential host responses and influences the expression of other regulatorily linked virulence factors (Figures 2–4). Our findings have important consequences for recently proposed antivirulence therapies, targeting pyoverdine-mediated iron uptake (Bonchi et al., 2014, 2015; Imperi et al., 2013; Kaneko et al., 2007; Ross-Gillespie et al., 2014), because complex interactions between bacterial load, host response and regulatory pleiotropy could result in unpredictable treatment outcomes (García-Contreras et al., 2014). We examined this possibility for our system and found an overall positive relationship between pyoverdine availability and virulence, but also notable deviations from a monotonic pattern. For instance, the supplementation of low levels of pyoverdine significantly decreased rather than increased virulence, with this treatment reaching virulence levels comparable to infections with the pyoverdine knockout strain (Figure 5).

Some of the discovered complex nonlinear associations between bacterial load, host response, pleiotropy and virulence warrant closer examination. For instance, why does increased pyoverdine availability (50  $\mu\text{M}$  and 250  $\mu\text{M}$  supplementation regimes) increase virulence despite the fact that these treatments reduce bacterial growth in



vivo (Figure 2) and results, in vitro at least, in the downregulation of the coupled virulence factor exotoxin A (Figure 4)? One possible explanation is that high pyoverdine supplementation triggers an excessive host response, which is not only curbing bacterial growth, but is also damaging the host itself. For instance, *G. mellonella* produces the iron chelator transferrin as part of its innate immune response (Han, Nam, Seo, & Yun, 2004), a protein which actively counteracts the iron-scavenging activities of pathogens (Miethke & Marahiel, 2007). Such a host response typically entails costs in terms of metabolic burden and autoimmune damage, and therefore must be appropriately calibrated (Day, Graham, & Read, 2007; Medzhitov, Schneider, & Soares, 2012). An overreaction from the host, perhaps in response to a high concentration of pyoverdine, could actually exacerbate, rather than reduce, virulence. Important to note is that although pyoverdine seems to induce a host response (Figure 3), it is not toxic itself, as larvae infected with pyoverdine alone all remained healthy (Figure 5).

Another complex association was that when increasing pyoverdine availability a little bit (10  $\mu$ M) compared to the wild-type treatment, we observed a significant reduction of virulence (Figure 5). This drop can potentially be explained by a host response too, but this time by a well-calibrated one, which primarily harms the pathogen while being beneficial for the host. If this explanation holds true, then the supplementation of moderate amounts of pyoverdine could represent a treatment that boosts host tolerance. Interestingly, treatments that increase host tolerance have, in addition to antivirulence approaches, been proposed as alternative ways to combat infections (Ayres & Schneider, 2012; Medzhitov et al., 2012; Vale et al., 2014, 2016).

Finally, we observed that infections with intermediate amounts of gallium (50  $\mu$ M) were significantly less virulent than infections with the pyoverdine-deficient knockout strain (Figure 5). This suggests that this treatment has other effects, in addition to simply depriving siderophores from pathogens. One explanation would be that gallium has some general toxicity towards bacteria beyond its role in inhibiting iron uptake (Bonchi et al., 2014). An alternative explanation, which is supported by our previous findings (Ross-Gillespie et al., 2014) but also the qPCR data (Figure 4), is that intermediate gallium levels maintain pyoverdine synthesis, while high gallium levels completely stall the production. This steady production likely imposes a twofold cost on bacteria: gallium does not only prevent pyoverdine-mediated iron uptake, but also induces continuous replacement of pyoverdine, which likely demands a high metabolic investment for very little reward (because pyoverdine is quenched by gallium once secreted). Given the ubiquity of linkages and feedback loops in the genetic architecture of bacteria (Dumas, Ross-Gillespie, & Kümmerli, 2013; Fazli et al., 2014; García-Contreras et al., 2014; Nadal Jimenez et al., 2012), such features are likely important contributors to nonadditive effects between pathogen behaviour, fitness and virulence.

Given the complexities of host-pathogen relationships we have highlighted in this study, what could be the evolutionary consequences for antivirulence therapies? The central tenet of this approach was that disarming rather than killing pathogens should induce weaker selection for resistance because it exerts only minimal effects

on pathogen fitness (André & Godelle, 2005; Pepper, 2008; Rasko & Sperandio, 2010; Stanton, 2013). Our study demonstrates that antivirulence approaches can in fact substantially modulate pathogen fitness (Figure 2; see also Liu et al., 2008), which clearly offers natural selection the opportunity to favour pathogen variants that are partially or fully resistant to the treatment (see Maeda et al., 2012; Ross-Gillespie et al., 2014; Allen et al., 2014; for detailed discussion). One obvious evolutionary response of pathogens in response to virulence factor quenching is to overproduce the virulence factor in question in order to outpace the quenching activity of the drug. Our results indicate that such an adaptation could affect the host in two different ways. If the increase in virulence factor production is substantial, this could lead to the evolution of a more virulent pathogen, which causes increased damage to the host in the absence of the treatment. Conversely, if the increase in virulence factor production is relatively small, then it could positively stimulate host responses, which in turn could curb virulence. Evolutionary responses leading to increased virulence factor production would likely involve the modification of regulatory elements. As evidenced by our study, regulatory elements can not only affect the expression of the targeted virulence factor, but also modify the expression of additional linked virulence factors in the same regulatory network (see Figure 4). How exactly such regulatory linkage would alter global virulence factor expression profiles of a pathogen in a host and how this feeds back on virulence cannot easily be foreseen, and might vary in response to the specific host stimuli present in an infection (Park et al., 2014). Finally, there might also be variation between host individuals regarding the extent to which they can cope with altered virulence factor expression. Conceivably, for an immunocompromised host even a minor overexpression of virulence factors might be fatal, whereas a healthy host might be more tolerant and easily able to cope with higher virulence factor levels. Taken together, our considerations show that we still have very limited understanding of the evolutionary consequences of antivirulence therapies. There is definitely a great need for controlled experimental evolution studies that measure selection pressures, adaptation patterns and host responses at both the proximate and ultimate level.

Given our dwindling supply of new antimicrobials, and the increasing prevalence of resistance to those we already have (Fischbach & Walsh, 2009; Levy & Marshall, 2004), creative approaches such as antivirulence therapies are certainly required (Perron, Inglis, Pennings, & Cobey, 2015; Ross-Gillespie & Kümmerli, 2014). To turn these ideas into effective and robust clinical therapies, however, we must delve deeper into the complexity of host-pathogen systems.

## ACKNOWLEDGEMENTS

We thank Martina Lardi for technical assistance and three anonymous reviewers for constructive comments. RK was supported by the Swiss National Science Foundation (grant no. PP00P3-139164) and the Novartis Foundation for medical-biological research (14B0457); MW was supported by DAAD; and SPB was supported by HFSP (grant RGP0011/2014).

# 4 Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections

## DATA ARCHIVING STATEMENT

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8ks41>

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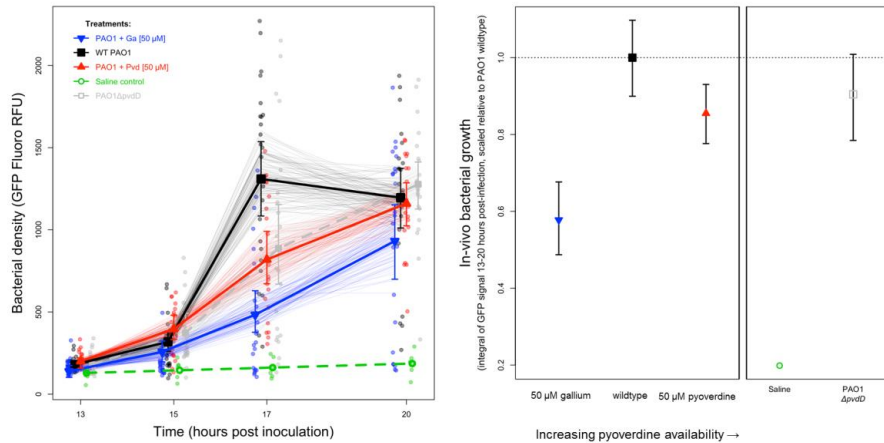
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## SUPPORTING INFORMATION

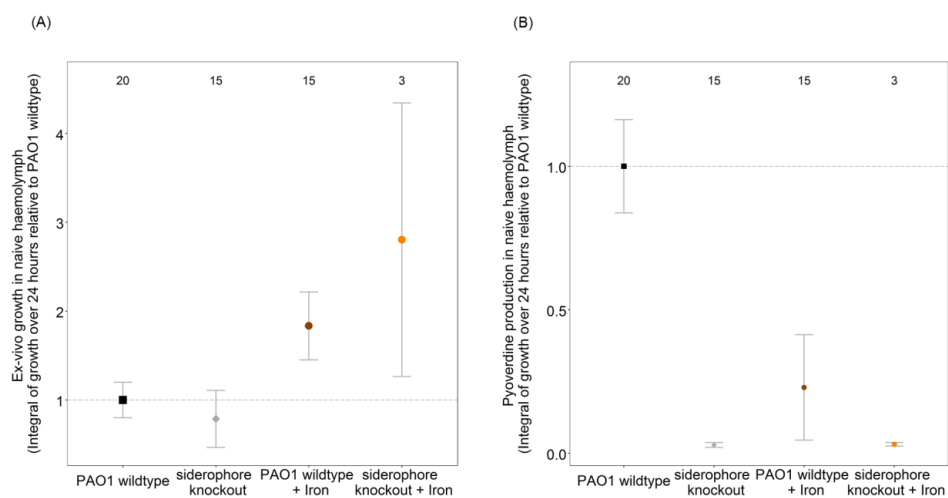
Additional Supporting Information may be found online in the supporting information tab for this article.

## 4.1 Supporting Material



**Figure S1.** Growth trajectories of *P. aeruginosa* within *G. mellonella* larvae confirm that gallium and pyoverdine supplementation both significantly reduced bacterial growth compared to the unsupplemented wildtype (permutation test with 10,000 iterations:  $p = 0.030$ ). *In vivo* bacterial density was estimated from constitutively expressed GFP signal in host homogenates (points). This involved destructively sampling up to 96 larvae per treatment (~24 per time point; or  $n = 6$  for the saline control). Because we were unable to track infections within an individual through time, we used bootstrap resampling of our observed data to generate replicated sets of estimated trajectories, a random sample of which are shown (faint lines). Symbols and error bars denote the medians, 2.5% and 97.5% quantiles from  $n = 10,000$  bootstrap-replicated datasets. We fitted splines to each trajectory and summarized the overall growth patterns using areas-under-curves. These resulting distributions of these growth integrals are given in the final plot (medians with 2.5% and 97.5% quantiles).

## 4.1 Supporting Material



**Figure S2** Growth (A) and pyoverdine production (B) of the wildtype strain and the pyoverdine-deficient mutant in naive haemolymph extracted from *G. mellonella* larvae. (A) The supplementation of 100  $\mu\text{M}$   $\text{FeCl}_3$  to the haemolymph significantly increased the growth of both the wildtype strain and the pyoverdine-deficient mutant, demonstrating that iron is a growth-limiting factor in the host environment. The observed pyoverdine-production profiles confirmed this assertion (B). Specifically, the wildtype strain produced high amounts of pyoverdine in the unsupplemented haemolymph, but reduced its investment to baseline level when iron was added to the haemolymph. Numbers on top show sample size of each treatment.

## 4 Manipulating Virulence Factor Availability Can Have Complex Consequences for Infections

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Gene	Sequence (5' → 3')
<i>pvdS</i> (forward)	AGG AAG AAG GCC TGA ACG TG
<i>pvdS</i> (reverse)	CCT TGG CGA TGT CCT TCT GT
<i>pvdA</i> (forward)	TGT TCC ACC ACA GCC AGT AC
<i>pvdA</i> (reverse)	GGG TAG CTG TCG TTG AGG TC
<i>toxA</i> (forward)	AAA AGC GCT GGA GCG AAT GG
<i>toxA</i> (reverse)	GGG AAA TGC AGG CGA TGA CTG AT
<i>prpL</i> (forward)	TCT ACA ACA CCA CCC AGT GC
<i>prpL</i> (reverse)	TTG CCC TGC GAG TAC TTC TT
<i>rpoD</i> (forward)	GGG GAT CAA CGT ATT CGA GA
<i>rpoD</i> (reverse)	ATC GAT ATA GCC GCT GAG GA

S1 Table. Genes and primers used for qPCR. We studied four genes involved in pyoverdine-mediated signaling, which are *pvdS* (encoding the iron-starvation sigma factor), *pvdA* (coding for an enzyme critical for pyoverdine synthesis), *toxA* (coding for exotoxin A), and *prpL* (encoding protease IV). Primers were designed based on sequences from the *Pseudomonas* genome database ([www.pseudomonas.com](http://www.pseudomonas.com)) using the Primer3Plus platform aiming for 200 bp amplicons.

# The Physical Boundaries of Public Goods Cooperation Between Surface-Attached Bacterial Cells

This research was originally published in Proceedings of the Royal Society B

**The physical boundaries of public goods cooperation between surface-attached bacterial cells**

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doi: 10.1098/rspb.2017.0631

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## **Abstract**

Bacteria secrete a variety of compounds important for nutrient scavenging, competition mediation and infection establishment. While there is a general consensus that secreted compounds can be shared and therefore have social consequences for the bacterial collective, we know little about the physical limits of such bacterial social interactions. Here, we address this issue by studying the sharing of iron-scavenging siderophores between surface-attached microcolonies of the bacterium *Pseudomonas aeruginosa*. Using single-cell fluorescent microscopy, we show that siderophores, secreted by producers, quickly reach non-producers within a range of 100  $\mu\text{m}$ , and significantly boost their fitness. Producers in turn respond to variation in sharing efficiency by adjusting their pyoverdine investment levels. These social effects wane with larger cell-to-cell distances and on hard surfaces. Thus, our findings reveal the boundaries of compound sharing, and show that sharing is particularly relevant between nearby yet physically separated bacteria on soft surfaces, matching realistic natural conditions.



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## Introduction

The study of cooperative interactions in bacteria is of interdisciplinary interest, as it is relevant for understanding microbial community assembly (1, 2), the establishment of infections (3-5), and biotechnological processes (6). Bacteria exhibit a wide range of cooperative traits, including the formation of biofilms and fruiting bodies, the secretion of toxins to infect hosts, coordinated swarming, and the scavenging of nutrients from the environment through the secretion of shareable compounds, such as enzymes and siderophores (7,8). While the existing body of work has greatly changed our perception of bacteria - from simple autarkic individuals to sophisticated organisms, interacting and cooperating with each other - there are still considerable knowledge gaps. For instance, many of the insights gained on the sharing of public goods are based on experiments in planktonic batch cultures, where behavioural responses are averaged across millions of cells. This contrasts with the natural lifestyle of bacteria, where individual cells adhere to surfaces and form biofilms, and primarily interact with their immediate neighbours at the micrometre scale (9,10). The mismatch between laboratory and natural conditions has led to controversies in the field regarding the general relevance of microbial cooperation (11-13).

In our paper, we tackle these issues by testing whether and to what extent secreted siderophores are shared between surface-attached individuals of the bacterium *Pseudomonas aeruginosa* using fluorescent microscopy. Siderophores are secondary metabolites produced by bacteria to scavenge iron from the environment, where it typically occurs in its insoluble ferric form or is actively withheld by the host in the context of infections (14, 15). In our experiments, we examined the production and secretion of pyoverdine, the main siderophore of *P. aeruginosa* (16). Pyoverdine production has become a model trait to study cooperation in bacteria, because it fulfils all the criteria of a cooperative trait:

it is costly to produce and secreted outside the cell, where it generates benefits in iron-limited media for the producer itself, but also for nearby individuals with a compatible receptor (17–19). Although highly influential, many of the insights gained are based on batch culture experiments, which tell us little about whether pyoverdine is also shared in surface-attached communities, where molecule diffusion might be limited, and thus the range of sharing constrained (13, 20). However, such knowledge is key to understand whether public goods cooperation occurs in natural settings and in infections, where bacteria typically live in biofilms attached to organic and inorganic substrates (8, 21).

Here, we present data from fluorescence time-laps microscopy experiments that examined bacterial interactions in real time at the micrometer scale. First, we tested whether pyoverdine molecules, secreted by producing cells, reach individuals that cannot produce pyoverdine themselves but have the receptor for uptake. Such evidence would be a direct demonstration of molecule sharing. Second, we test whether pyoverdine serves as a signalling molecule (22), allowing producers to respond to changes in their social neighbourhood. Specifically, we predict that lower pyoverdine investment is required in a cooperative neighbourhood due to the efficient reciprocal pyoverdine sharing. Conversely, non-producers, which act as a sink for pyoverdine, should trigger increased investment levels to compensate for pyoverdine loss (23,24). Third, we examined whether pyoverdine diffusivity limits the range across which pyoverdine can be efficiently shared. To this end, we manipulated both the media viscosity, which directly affects molecule diffusion, and the distance between producer and non-producer cells, which increases the diffusion time and reduces the amount of pyoverdine reaching non-producers. Finally, we used time-laps microscopy to quantify fitness effects of pyoverdine production and sharing in growing microcolonies. Taken together, our experiments shed light on the physical boundaries and individual fitness consequences of public goods sharing.

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## Results

### Pyoverdine diffuses from producer to non-producers

We put mono- and mixed cultures of the wildtype strain PAO1 and its isogenic pyoverdine mutant PAO1  $\Delta pvdD$  (tagged with a fitness-neutral mCherry marker) on iron-limited agarose pads on a sealed microscopy slide. Cultures were highly diluted such that single cells were physically separated from each other at the beginning of the experiment. We then monitored the pyoverdine fluorescence in growing micro-colonies over time for both strains under the microscope. Pyoverdine fluorescence becomes visible in the periplasma, where molecule maturation occurs (13, 32) (figure 1b). We found that fluorescence in non-producer colonies was indistinguishable from background signal one hour after incubation, indicating that no detectable pyoverdine had yet been taken up (figures 1a+c and S1). However, pyoverdine fluorescence in non-producer cells significantly increased over time in mixed cultures (LM:  $F_{5,7567} = 913$ ,  $p < 0.001$ ) was significantly higher than the background fluorescence in non-producers growing as monocultures (t-test:  $t_{3945} = 79.33$ ,  $p < 0.001$ , figures 1a+d and S1). This demonstrates that significant amounts of pyoverdine diffuse from producer to non-producer microcolonies even when there is no direct cell-to-cell contact.

### Producers alter pyoverdine investment in the presence of non-producers

To test whether producers respond to changes in their social environment, we followed the expression pattern of *pvdA* (a gene involved in pyoverdine synthesis) and natural pyoverdine fluorescence in growing producer microcolonies (figures 2 and S2). In our control treatment with added iron, both *pvdA* and pyoverdine signal were downregulated compared to iron-limited conditions,

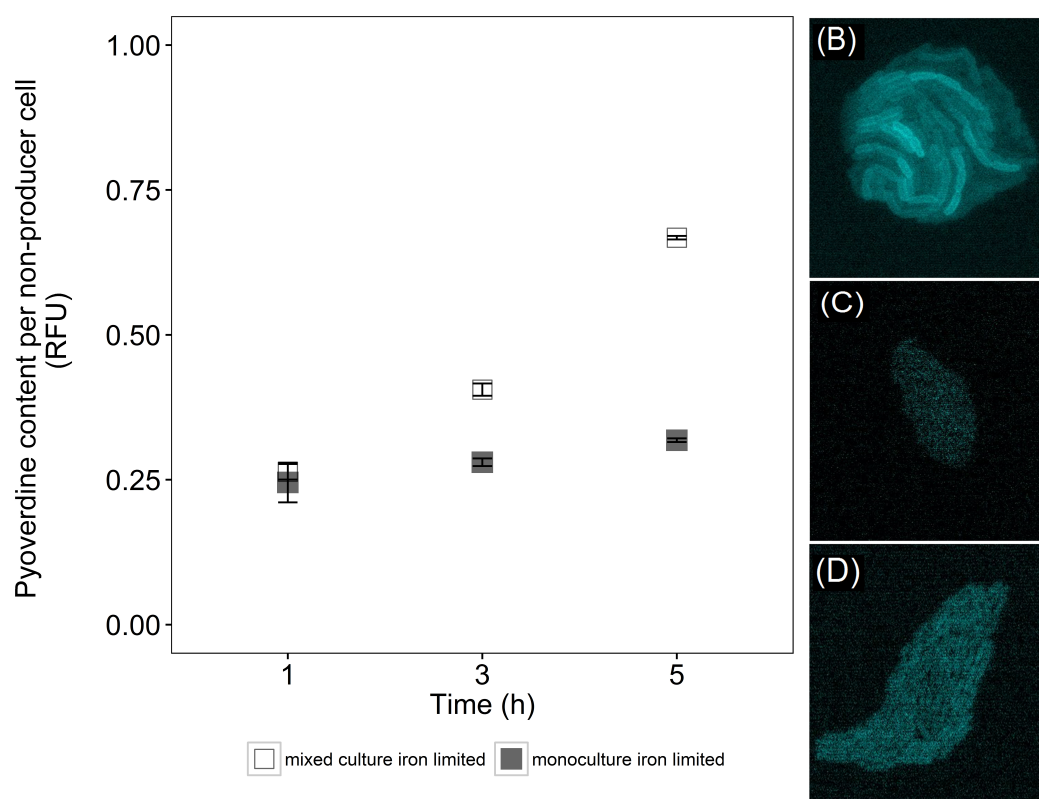
demonstrating the functioning and high sensitivity of our reporters. Under iron limitation, meanwhile, *pvdA*-expression was significantly higher in mixed compared to monoculture at one hour (t-test:  $t_{115} = 5.23$ ,  $p < 0.001$ ) and three hours ( $t_{860} = 13.92$ ,  $p < 0.001$ ) post-incubation (figures 2a and S2a). Pyoverdine fluorescence mirrored *pvdA* expression patterns, with higher pyoverdine levels being detected in producer cells growing in mixed cultures (figures 2b and S2b), although the difference was only significant after three hours (t-test:  $t_{992} = 13.30$ ,  $p < 0.001$ ), but not after one hour (t-test:  $t_{88} = 1.26$ ,  $p = 0.211$ ). The picture changed five hours post-incubation, where both *pvdA*-expression and pyoverdine fluorescence were significantly lower in mixed compared to monocultures (*pvdA*-expression:  $t_{6441} = -16.67$ ,  $p < 0.001$ ; pyoverdine fluorescence:  $t_{6017} = -50.01$ ,  $p < 0.001$ ). These analyses demonstrate that producers rapidly alter pyoverdine investment in response to the presence of non-producers.

### **Pyoverdine non-producers outgrow producers in mixed cultures**

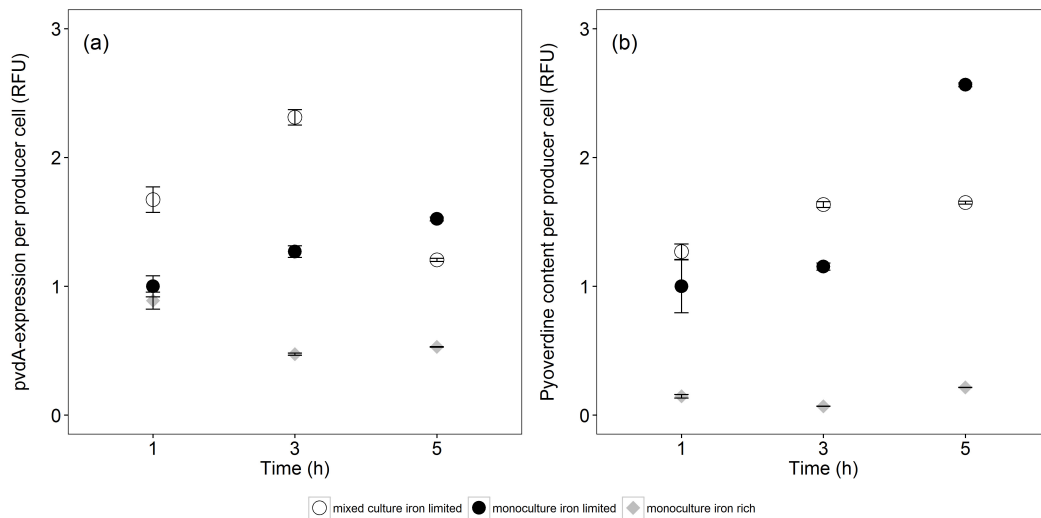
After having established that pyoverdine is shared between neighbouring, yet physically separated surface-attached microcolonies, we explored the fitness consequences of pyoverdine sharing. This is important because experiments in liquid batch cultures repeatedly revealed that non-producers can outcompete producers, by saving the cost of pyoverdine production, yet exploiting the siderophores produced by others, a phenomenon that is called "cheating" (17, 33-36). To examine whether cheating is also possible when bacteria grow as surface-attached microcolonies, we grew producers and non-producers in mono and mixed culture and followed microcolony growth dynamics over time (figure 3). Control experiments in iron-supplemented media revealed that all strains grew equally well regardless of whether they grew in mono or mixed cultures (figure S4). In iron-limited media, however, we found that microcolony growth was significantly reduced for non-producers compared to producers

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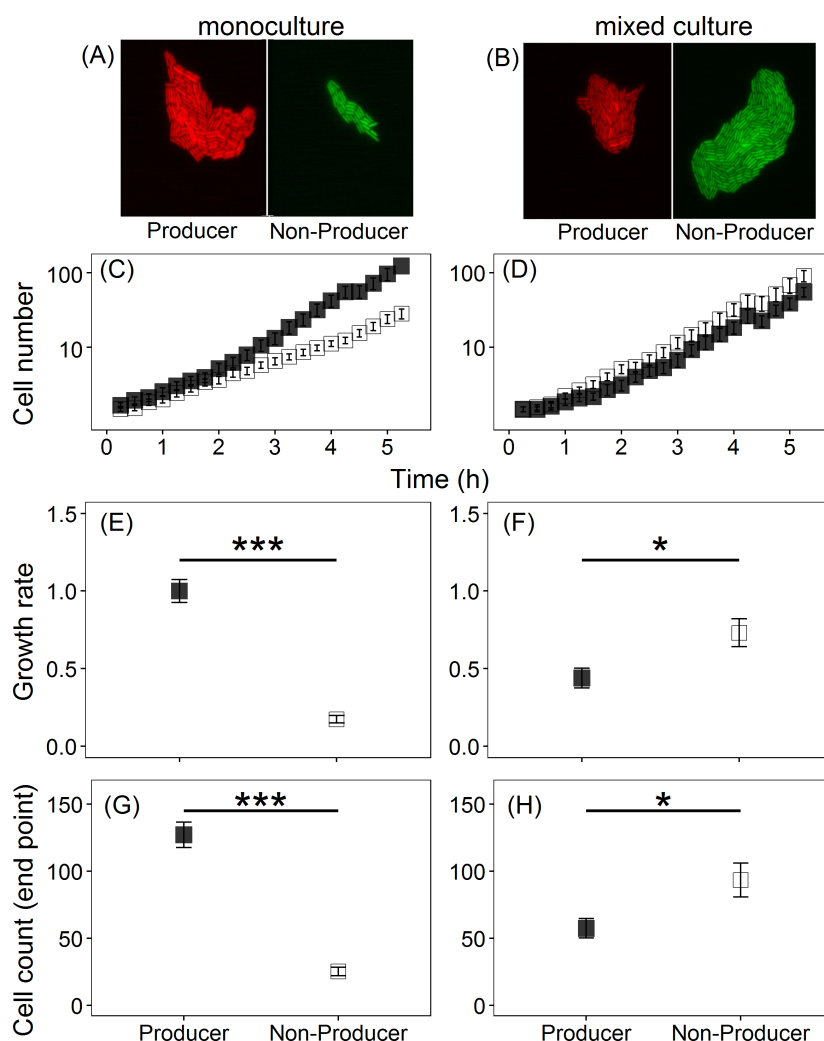
(growth rate:  $t_{23} = -10.57$ ,  $p < 0.001$ , figure 3e; cell number:  $t_{23} = -10.27$ ,  $p < 0.001$ , figure 3g). This shows that the inability to produce pyoverdine is a major handicap in iron-limited media.



**Figure 1:** Pyoverdine is taken up by non-producing cells in a time-dependent manner, demonstrating pyoverdine sharing between physically separated, surface-attached microcolonies of *P. aeruginosa*. (a) Time-course measures on natural pyoverdine fluorescence units (RFU) shows constant background fluorescence in non-producer cells grown in monocultures (filled squares), whereas pyoverdine fluorescence significantly increased in non-producer cells grown in mixed cultures with producers (open squares). Mean relative fluorescence values  $\pm$  standard errors are scaled relative to producer monocultures after one hour of growth. Representative microscopy pictures show pyoverdine fluorescence in a producer microcolony (b), a non-producer colony from a monoculture (c), and a non-producer colony from a mixed culture (d). Important to note is that only apo-pyoverdine (i.e. iron-free pyoverdine) is fluorescent, and therefore the measured fluorescence intensities represent a conservative measure of the actual pyoverdine content per cell. Furthermore, the fluorescence intensity in producer cells is always higher than in non-producer cells because it represents the sum of pyoverdine uptake and newly synthesized pyoverdine, whereas for non-producers, fluorescence represents pyoverdine uptake only.



**Figure 2:** Producer cells adjust their pyoverdine investment level in response to changes in the social environment. (a) Time-course data show that *pvdA*, a gene encoding an enzyme involved in pyoverdine synthesis, is down-regulated in iron-rich media (grey diamonds), but up-regulated in iron-deplete media. Importantly, producers exhibited different *pvdA* expression patterns depending on whether they grew together with non-producers (open circles) or as monoculture (filled circles). While producers showed increased gene expression in mixed compared to monoculture after one and three hours, the pattern flipped after five hours. (b) The same qualitative pattern was observed when measuring pyoverdine content per cell, as relative fluorescence units (RFU). Fluorescence values are scaled relative to the producer monocultures after one hour of growth. Error bars indicate standard errors of the mean.



**Figure 3:** Growth performance of surface-attached microcolonies of pyoverdine producers (filled squares) and non-producers (open squares) of *P. aeruginosa* in monocultures (left column) and mixed cultures (right column). While pyoverdine non-producers show growth deficiencies in monoculture, due to their inability to scavenge iron, they outcompete the producers in mixed cultures. This growth pattern shows that non-producers save costs by not making any pyoverdine, yet gain fitness benefits by capitalizing on the pyoverdine secreted by the producers. (a) and (b) show representative microscopy pictures for monocultures and mixed cultures, respectively. The overall growth trajectories of producers and non-producers differ substantially between monocultures (c) and mixed cultures (d). While producers had a significantly higher growth rate (e) and grew to higher cell numbers (g) in monocultures, the exact opposite was the case in mixed cultures for both the growth rate (f) and cell number (h). Growth parameters are given relative to the producers in monoculture. Asterisks indicate significant differences and error bars denote standard errors of the mean.

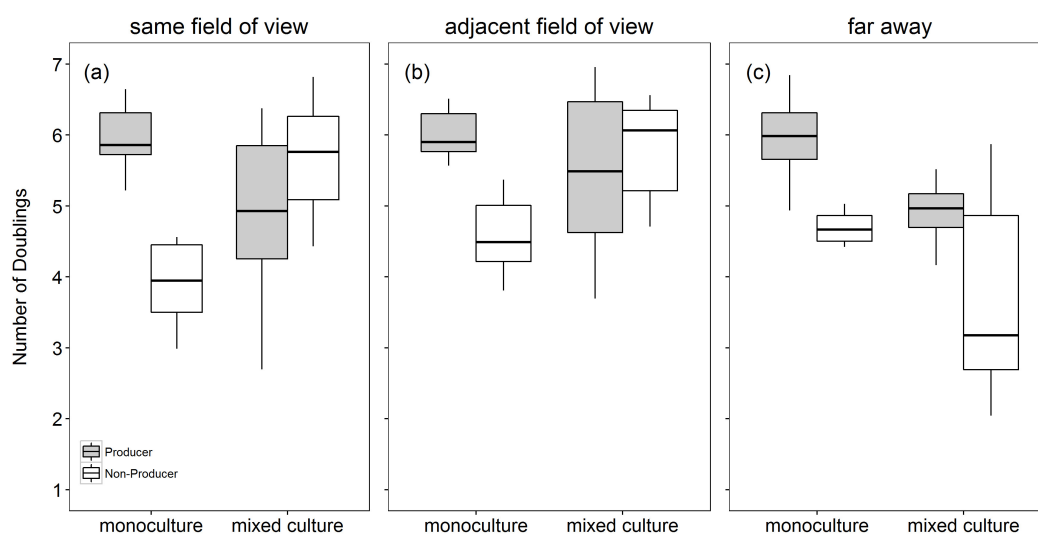


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This fitness pattern diametrically flipped in mixed cultures, where non-producer microcolonies grew significantly faster ( $t_{35} = 2.64$ ,  $p = 0.012$ , figure 3f) and to higher cell numbers ( $t_{31} = 2.48$ ,  $p = 0.019$ , figure 3h) than producer microcolonies. Intriguingly, non-producers experienced a relative fitness advantage between hours one and three (t-test:  $t_{20} = 4.53$ ,  $p < 0.001$ ), but not at later time points ( $t_{41} = -0.184$ ,  $p = 0.855$ ; figure S5). This specific period, at which the relative fitness advantage manifests, perfectly matches the timeframe during which producers exhibited highest *pvdA* expression levels, and non-producers started accumulating pyoverdine (figure 2 and S2). Our findings thus provide a direct temporal link between the high costs of pyoverdine investment to producers, the increased benefits accruing to non-producers, and the resulting opportunity for non-producers to act as cheaters and to successfully outcompete producers.

### **The physical boundaries of pyoverdine sharing and benefits for non-producers**

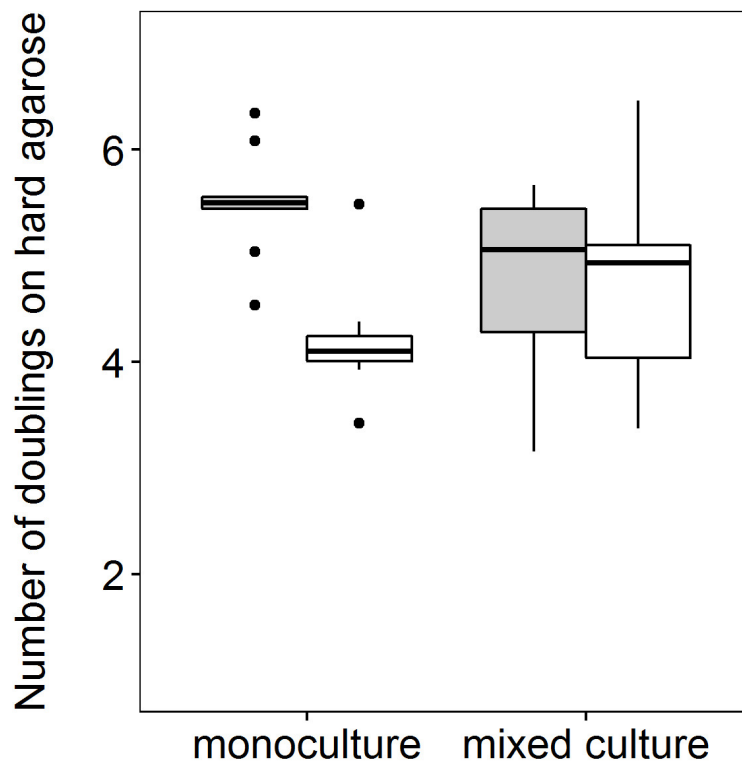
The above experiments revealed that pyoverdine can be shared between two physically separated microcolonies when grown in the same field of view ( $128 \times 96 \mu\text{m}$ ) under the microscope (average  $\pm$  SD distance between cells  $d = 36.2 \pm 18.2 \mu\text{m}$ ). Next, we asked what the physical limit of pyoverdine sharing is. We thus repeated the above experiment, but this time we focussed on non-producer cells that had no producer cell within the same field of view, but only a more distant producer in an adjacent field of view (minimal distance  $d = \text{ca. } 100 \mu\text{m}$ ). Under these conditions, we found that non-producers benefited from the presence of more distant producers in the same way as they benefited from the presence of a close producer (figure 4a+b; significantly increased growth of non-producers in mixed culture, for  $d = \text{ca. } 100$ , t-test:  $t_{14} = 4.02$ ,  $p = 0.001$ ). However, contrary to the previous observation (figure 4a), the producer no longer experienced a significant growth reduction in the presence of a more distant non-producer (figure 4b, for  $d = \text{ca. } 100 \mu\text{m}$ ,  $t_9 = -0.80$ ,  $p = 0.442$ ). We



**Figure 4:** The relative fitness advantage of pyoverdine non-producers in mixed cultures is dependent on the distance between producer (grey) and non-producer (white) microcolonies. In monoculture assays, the non-producers had significantly lower number of doublings than the producers in all experiments. In mixed cultures, meanwhile, the number of doublings of non-producers significantly increased when the producer microcolony was (a) within the same field of view (average distance between cells 36  $\mu\text{m}$ ), (b) in an adjacent field of view (minimal distance ca. 100  $\mu\text{m}$ ), but not when producers were far away (on opposite ends of the agarose pad) (c). These analyses show that pyoverdine can be shared and exploited across a relatively large distance. Boxplots represent the median with 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers show the 1.5 IQR.

then expanded the distance between non-producers and producers even further by adding the two strains on opposite ends of a double-sized agarose pad. In contrast to the previous results, this assay revealed that non-producers had significantly lower number of doublings in both mixed ( $t_{13} = -2.41$ ,  $p = 0.032$ ) and monocultures ( $t_9 = -4.66$ ,  $p = 0.001$ ) (figure 4c), suggesting that pyoverdine diffusion and sharing is disabled across this large distance in the time frame analysed.

In addition, our microscopy experiment revealed that pyoverdine sharing did not only affect the doubling rate of cells but also their size (figure S6). While non-producer cells were significantly smaller than producer cells in monocul-



**Figure 5:** *Pyoverdine sharing is impeded on hard surfaces. While the previous experiments showed that pyoverdine is extensively shared between neighbouring microcolonies on relatively soft surfaces (1% agarose), efficient sharing was no longer possible on hard surfaces (2% agarose) even when non-producers (open squares) were located within the same field of view (filled squares). Boxplots represent the median with 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers show the 1.5 IQR*

ture (LM:  $F_{1,1294} = 150.90$ ,  $p < 0.001$ , measured three hours post-incubation), the cell size of non-producers significantly increased when grown together with a nearby producing neighbour (same field of view  $d = \text{ca. } 36 \mu\text{m}$ :  $t_{446} = 10.24$ ,  $p < 0.001$ , figure S6 a; adjacent field of view  $d = \text{ca. } 100 \mu\text{m}$ :  $t_{161} = 4.10$ ,  $p < 0.001$ , figure S6 b), but not when producers were far away (on opposite ends of the agarose pad:  $t_{263} = 0.45$ ,  $p = 0.660$ , figure S6 c).

While the above experiments examined pyoverdine sharing on 1% agarose pads - a solid yet still moist environment - we were wondering whether pyoverdine sharing is also possible on much harder and drier surfaces. To test this possibility, we repeated the growth experiments on 2% agarose pads. Un-

der these conditions, we observed that non-producers no longer benefited from growing next to producers (no significant difference in the doubling numbers between mono and mixed cultures:  $t_{14} = -0.98$ ,  $p = 0.346$ ) (figure 5). This finding is compatible with the view that molecule diffusion is much reduced on very hard surfaces, preventing pyoverdine sharing between adjacent microcolonies.

## Discussion

Our single-cell analysis on pyoverdine production in *P. aeruginosa* provides several novel insights on the social interaction dynamics between surface-attached bacteria. First, we found that pyoverdine secreted by producer cells is taken up by physically separated non-producer cells, thereby directly demonstrating pyoverdine sharing. Second, we discovered that producer cells rapidly adjust pyoverdine expression levels when non-producers are nearby, by first up-regulating and then down-regulating pyoverdine investment. Third, we demonstrate that pyoverdine sharing has fitness consequences, as it boosts the growth and cell size of non-producers when growing in the vicinity of producers. Finally, we explored the physical limits of pyoverdine sharing and show that on soft surfaces, pyoverdine can be shared across a considerably large scale (at least 100  $\mu\text{m}$ , i.e. ca. 50 times the length of a bacterium), whereas efficient sharing is impeded with larger distances between cells and on hard surfaces. Altogether, our experiments suggest that public goods sharing and exploitation can take place between surface-attached bacteria across a wide range of naturally relevant conditions, and is mediated by molecule diffusion without the need for direct cell-to-cell contact.

Our results oppose previous work claiming that pyoverdine is predominantly shared between adjacent cells within the same microcolony (13). This claim has provoked a controversy on whether pyoverdine, and secreted compounds in

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general, can indeed be regarded as public goods (12, 37). The difference between our experiments and the ones performed by Julou et al. (13) is that their study solely examined pyoverdine content of cells within the same microcolony. Unlike in our study, there was no direct test of whether pyoverdine diffuses to neighbouring microcolonies and what the fitness consequences of such diffusion would be. While we agree that a considerable amount of pyoverdine is probably shared within the microcolony, we here demonstrate that a significant amount of this molecule also diffuses out of the microcolony, providing significant growth benefits to physically separated neighbouring microcolonies. Thus, our work concisely resolves the debate by showing that secreted hydrophilic compounds, such as pyoverdine (38), can be considered as public goods, even in structured environments, with the amount of sharing and the associated fitness consequences being dependent on the distance between neighbouring microcolonies. Moreover, the distance effect we report here at the single-cell level is in line with density effects described at the community level, where secreted compounds are predominantly shared and become exploitable at higher cell densities (i.e. when cell-to-cell distance is reduced 39-42).

A key advantage of single-cell analyses is that they allow the tracking of bacterial behavioural and growth changes in real time with high precision, immediately after the start of an experiment. This contrasts with batch culture experiments, where responses can only be measured after several hours, once the proxies for responses (e.g. optical density) become detectable at the population level. For instance, results from previous batch-culture studies suggest that pyoverdine producers seem to overinvest in pyoverdine when grown together with non-producers (23, 24). However, the interpretation of these results were based upon a number of assumptions, and the batch-culture approach precluded an in-depth analysis of the temporal pattern and consequences of such overinvestment. Our analysis now provides a nuanced view on the interactions

between producers and non-producers. We could show that soon after the inoculation of bacteria on the agarose pad, producers started overexpressing pyoverdine (figures 2 and S2), which coincided with pyoverdine accumulation in non-producer cells (figures 1 and S1), and significant fitness advantages to non-producers (figure S5). Moreover, our findings indicate that producers can possibly respond to exploitation by down-regulating pyoverdine production at later time points, a response that correlated with the abolishment of further fitness advantages to non-producers.

Our considerations above raise questions regarding the regulatory mechanisms involved in controlling the observed expression changes. Molecular studies suggest that pyoverdine serves as a signalling molecule regulating its own production (22, 43). Specifically, when iron-loaded pyoverdine binds to its cognate receptor FpvA, a signalling cascade is triggered, which results in the release of PvdS (the iron-starvation sigma factor, initially bound to the inner cell membrane by the anti-sigma factor FpvR). PvdS then upregulates pyoverdine production. This positive feedback, triggered by successful iron uptake, is opposed by a negative feedback operated by Fur (ferric uptake regulator), which silences pyoverdine synthesis once enough iron has been taken up (16, 44). Our results can be interpreted in the light of these feedbacks, given that the relative strength of the opposing feedbacks determines the resulting pyoverdine investment levels (45). For example, producer micro-colonies reach higher cell densities in mono-compared to mixed cultures (figure 3, after 3h:  $13.2 \pm 2.3$  versus  $6.7 \pm 1.3$  cells; after 5h:  $122.7 \pm 17.9$  versus  $55.0 \pm 8.1$  cells, respectively). Higher cell densities likely lead to more efficient pyoverdine sharing, which supposedly stimulates both pyoverdine-signalling and iron uptake. Positive and negative feedback should thus be in balance and result in an intermediate pyoverdine investment levels. Conversely, when producers grow in mixed cultures then cell density is reduced and non-producers serve as a sink

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for pyoverdine, thereby reducing iron supply to producers. In this scenario, the positive feedback should be stronger than the negative feedback, resulting in the upregulation of pyoverdine. While these elaborations are compatible with the pyoverdine expression patterns observed at hour one and three, the flip in expression patterns between mono and mixed cultures after five hours is more difficult to explain. One option would be that the previously described switch from pyoverdine production to recycling (46–48) occurs earlier in mixed than in monocultures. An alternative option would be that producers can recognize the presence of exploitative cheaters and downscale their cooperative efforts accordingly.

Our results showing that non-producers can outcompete producers in mixed cultures, even when microcolonies are physically separated, confirms predictions from social evolution theory for microbes (49-52). One key condition required for cooperation to be maintained is that cooperative acts must be more often directed towards other cooperators than expected by chance. This interaction probability is measured as the degree of relatedness  $r$ , a parameter central to inclusive fitness theory (53, 54). Traditionally, high relatedness has been associated with the physical separation of cooperators and non-cooperators into distinct patches (54). Our results now show that this traditional view is not necessarily applicable to public goods cooperation in bacteria, because the physical separation of pyoverdine producers and non-producers is insufficient to prevent exploitations and maintain cooperation (figure 3). Clearly, relatedness in our scenario should be measured at the scale at which pyoverdine sharing can occur (50), which exceeds the boundaries of a single microcolony. Thus, in scenarios where microbial cells are immobile, it is the diffusion properties of the public good that determines the degree of relatedness between interacting partners (49, 51).

In summary, our findings on pyoverdine sharing and exploitation between physically separated microcolonies has broad implications for our understanding of the social life of bacteria in many natural settings. This is because bacteria typically live in surface-attached communities in aquatic and terrestrial ecosystems, as well as in infections (8, 21). Many of these natural habitats feature soft surfaces, as mimicked by our experimental set up, making the diffusion and sharing of secreted compounds between cells highly likely. However, our work also reveals physical limits to public goods cooperation, namely on hard surfaces, where public good diffusion and sharing is impeded. This shows that whether or not a secreted compound is shared is context-dependent (38), and relies, amongst other factors, on the physical properties of the environment.

## Materials and Methods

### Strains and media

Our experiments featured the clinical isolate *P. aeruginosa* PAO1 (ATCC 15692), and its clean pyoverdine knock-out mutant (PAO1 $\Delta$ pvdD), directly derived from this wildtype. To be able to distinguish the two strains, we used fluorescent variants of these strains constructed via chromosomal insertion (*attTn7::ptac-gfp*, *attTn7::ptac-mcherry*) - i.e. PAO1-*gfp*, PAO1-*mcherry*, PAO1 $\Delta$ pvdD-*gfp* and PAO1 $\Delta$ pvdD-*mcherry*. A preliminary experiment revealed that these fluorescent markers did not affect the growth performance of the strains (Figure S2). For our gene expression experiments, we used the reporter strain PAO1pvdA-*gfp* (chromosomal insertion: *attB::pvdA-gfp*) (Kaneko et al. 2007). PvdA catalyses an important step in the biosynthesis pathway of pyoverdine (Leoni et al. 1996), and its expression level is therefore a good proxy for the investment into pyoverdine production.



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Overnight cultures were grown in 8 mL Lysogeny Broth (LB) medium in 50 mL Falcon tubes, and incubated at 37 °C, 200 rpm for 16-18 hours. Cells were then harvested by centrifugation at 3000 rpm for 3 minutes and resuspension in 8 mL of 0.8% NaCl (saline solution). For all experiments, we subsequently diluted the washed cultures in saline solution to an OD = 1 (optical density at 600 nm). For all microscopy experiments, we used CAA medium (per liter: 5 g casamino acids, 1.18 g  $K_2HPO_4 \cdot 3H_2O$ , 0.25 g  $MgSO_4 \cdot 7H_2O$ ). To create severe iron limitation, we added the chemical iron chelator 2,2-Bipyridine (final concentration 40  $\mu$ M). To create iron-replete conditions, we added 200  $\mu$ M  $FeCl_3$ . All chemicals were purchased from Sigma-Aldrich (Buchs SG, Switzerland).

## **Preparation of microscopy slides**

We adapted a method previously described in (de Jong et al. 2011). Standard microscopy slides (76 mm x 26 mm) were washed with EtOH and dried in a laminar flow. We used 65  $\mu$ L “Gene Frames” (Thermo Fisher Scientific) to prepare agarose pads. Each frame features a single chamber of 0.25 mm thickness (1.5 cm x 1.6 cm) and 65  $\mu$ L volume. The frame is coated with adhesives on both sides so that it sticks to the microscopy slide, and at the same time adheres the cover glass from the top. The sealed chamber is airproof, which is necessary to prevent evaporation and deformation of the pad during the experiment.

To prepare microscopy pads, we heated mL CAA supplemented with agarose (1% unless indicated otherwise) in a microwave. The melted agarose-media mix was subsequently cooled to approximately 50 °C. Next, we added the supplements: either 2,2-Bipyridine (final concentration 450  $\mu$ M) or  $FeCl_3$  (final concentration 200  $\mu$ M) to create iron-limited or iron-replete conditions, respectively. We pipetted 360  $\mu$ L of the agarose solution into the gene frame and immediately covered it with a cover glass. The cover glass was pressed down with a gentle pressure to dispose superfluous media. After the solidification of the agarose

pad (ca. 30 minutes), we removed the cover glass (by carefully sliding it sideways) and divided the original pad into 4 smaller pads of equal size by using a sterile scalpel. The further introduced channels between pads, which served as a reservoir for oxygen. We then put 1  $\mu\text{L}$  of highly diluted bacterial culture ( $\text{OD} = 1$  culture diluted by  $2.5 \cdot 10^4$ ) in the middle of each pad. Two pads were inoculated with a 1:1 mix of pyoverdine producers and non-producers, whereas the other two pads were inoculated with a monoculture (either producer or non-producer). After the inoculum drop had evaporated, we sealed the pads with a new cover glass using the adhesive of the Gene Frame. With this protocol, we managed to create agarose pads with consistent properties across experiments.

## **Microscopy setup and imaging**

All experiments were carried out at the Center for Microscope and Image Analysis of the University Zürich (ZMB) using a widefield Leica DMI6000 microscope. The microscope featured a plan APO PH3 objective ( $\text{NA} = 1.3$ ), an automated stage and an auto-focus. For fluorescent imaging, we used a Leica L5 filter cube for GFP (Emission:  $480 \text{ nm} \pm 40 \text{ nm}$ , Excitation:  $527 \text{ nm} \pm 30$ ,  $\text{DM} = 505$ ) and a Leica TX2 filter cube for mCherry (Emission:  $560 \text{ nm} \pm 40 \text{ nm}$ , Excitation:  $645 \text{ nm} \pm 75 \text{ nm}$ ). Auto-fluorescence of pyoverdine was captured with a Leica CFP filter cube (Emission:  $436 \text{ nm} \pm 20 \text{ nm}$ , Excitation:  $480 \text{ nm} \pm 40 \text{ nm}$ ,  $\text{DM} = 455$ ). We used a Leica DFC 350 FX camera (resolution:  $1392 \times 1040$  pixels) for image recording (16 bit colour depth).

## **Image processing and blank subtraction**

To extract information (cell size, fluorescence) from every single cell, images had first to be segmented. Segmentation is the process of dividing an image into objects and background. Since it is currently a bottleneck for high throughput image analysis (Van Valen et al. 2016), we developed a new workflow (see attached protocol for details). This workflow includes a protocol for the

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rapid, reliable and fully automated image segmentation without the need for any priors (i.e. information on cell size and shape) and manual corrections. The workflow starts with the machine learning, supervised object classification and segmentation tool ilastik (Sommer et al. 2011). Ilastik features a self-learning algorithm that autonomously explores the parameter space for object recognition. We used a low number of phase contrast images from our experiments to train ilastik for bacterial cell recognition and segmentation. Each training round is followed by user inputs regarding segmentation errors. These inputs are then incorporated in the next training round, until segmentation is optimized and error-free. Once the training is completed, microscopy images from all experiments can be fed to ilastik and segmentation is then carried out in a fully automated manner, without the need for any further manual corrections. Ilastik produces binary images as an output (black background vs. white objects).

For image analysis, this output was then transferred to Fiji, a free scientific image processing software package (Schindelin et al. 2012). We wrote specific macro-scripts in Fiji to fully automate the simultaneous analysis of multiple single-cell features such as cell size, shape, fluorescence, etc. First, we used the binary images to create an overlay of region of interests for every single cell, which could then be used in a second step to measure bacterial properties from phase contrast and fluorescence images (see supplementary material for a step-by-step protocol). Next, we applied a pixel-based blank correction procedure in Fiji, to obtain unbiased fluorescence intensities for each cell. For each agarose pad and time point, we imaged four empty random positions on the agarose pad without bacterial cells and averaged the grey values for each pixel. The averaged grey value of each pixel was then subtracted from the corresponding pixel position in images containing cells. This pixel-based blank correction accounts for intensity differences across the field of view caused by the optical properties of the microscope (vignetting). In the experiments where we simulta-

neously measured *pvdA-gfp* expression and pyoverdine fluorescence, we had to further correct for the leakage of pyoverdine signal into the GFP-channel. To do so, we imaged cells of the unmarked wildtype strain, which produced pyoverdine but had no GFP reporter. We then measured the pyoverdine signal in the GFP-channel at three different time points (one, three and five hours post-incubation), and then used these values to blank correct the fluorescence intensities in cells with the *pvdA-gfp* reporter.

### **Assays measuring *pvdA* expression and pyoverdine fluorescence**

To monitor pyoverdine investment by producer cells and pyoverdine uptake by non-producer cells, we quantified natural pyoverdine fluorescence in bacterial micro-colonies of both strains in mixed and monocultures over time. For producer micro-colonies we further measured *pvdA* expression levels over time. Because the excitation wavelength for pyoverdine fluorescence overlaps with the UV range, the high exposure time required to measure natural pyoverdine fluorescence induces damage (i.e. phototoxicity) to bacterial cells. Accordingly, each bacterial micro-colony could only be measured once. To obtain time course data for pyoverdine expression and uptake levels, we thus prepared multiple microscopy slides, as described above, and incubated them at 37 °C in a static incubator. At each time step (one, three and five hours post incubation), we then processed two slides for imaging. Exposure time for measuring GFP-fluorescence was 800 ms and for pyoverdine 1500 ms, with a (halogen) lamp intensity of 100%. To guarantee reliable automated image analysis, we only considered positions that were free from non-bacterial objects (e.g. dust) and where all cells laid within one focus layer. Focus was adjusted manually. We recorded at least five positions per treatment, time point and slide. The experiment was carried out twice, in two completely independent batches.

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## Fitness Assay

We used time-laps microscopy to measure the growth performance of pyoverdine producer cells (tagged with mCherry) and non-producer cells (tagged with GFP) in mixed and monoculture. As described above, we cut the agarose pad in four patches and inoculated two patches with a 1:1 mix of producers and non-producers, and one patch each with a monoculture. We then chose 20 positions (five per patch) that contained two separated cells (one cell of each strain for mixed cultures and two cells of the same type for monocultures), and imaged these positions sequentially every 15 minutes over 5 hours, using the automated stage function of the microscope. Following a position change and prior to imaging, we used the auto-focus function of the microscope to adjust the z-position in order to keep cells in focus. This protocol allowed us to follow the growth of micro-colonies from a single-cell stage.

We carried out the above fitness assays across a range of different conditions. In a control experiment, we added 200  $\mu\text{M}$   $\text{FeCl}_3$  to the agarose pad to study the strain growth in the absence of iron limitation. Since bacteria grow very well in iron-replete media, we stopped the imaging after three hours before micro-colonies started to grow in multiple layers. Next, we monitored strain growth on iron-limited 1% agarose pads supplemented with 450  $\mu\text{M}$  bipyridin. To examine whether pyoverdine sharing and fitness effects depend on the distance between two cells, we performed fitness assays where two cells were positioned: (i) close to one another in the same field of view (average distance between cells  $36.21 \mu\text{m} \pm 18.17 \text{ SD}$ ); (ii) further apart in adjacent fields of view (with an estimated minimum distance of 96  $\mu\text{m}$ , given the field of view size of 96  $\mu\text{m} \times 128 \mu\text{m}$ ); and (iii) far from one another. This latter condition was created by adding the two strains on opposite ends of an elongated double-sized agarose pad. Finally, we repeated the growth assays in media with increased

viscosity (i.e. on 2% agarose pads).

## **Statistical methods**

All statistical analyses were performed in R 3.3.0 (31) using linear models (ANOVA or t-tests). Prior to analysis, we used the Shapiro-Wilk test to check whether model residuals were normally distributed. Since each experiment was carried out in multiple independent experimental blocks, we scaled values within each block relative to the mean of the control treatment (i.e. pyoverdine producer monocultures). For all time-laps growth experiments, we considered the position (i.e. the field of view) as the level of replication. For the analysis of single cell fluorescence data, we considered each cell as a replicate.

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## **Acknowledgments**

We thank Urs Ziegler and Caroline Aemisegger for help with the microscope, Moritz Kirschmann for advice regarding single cell data analysis, and the SNSF (grant no. PP00P3\_165835 to RK), the ERC (grant no. 681295 to RK) and the DAAD (to MW) for funding.

## **Funding statement**

Funding organisations had no influence on study design, data collection and interpretation or the decision to submit the work for publication.

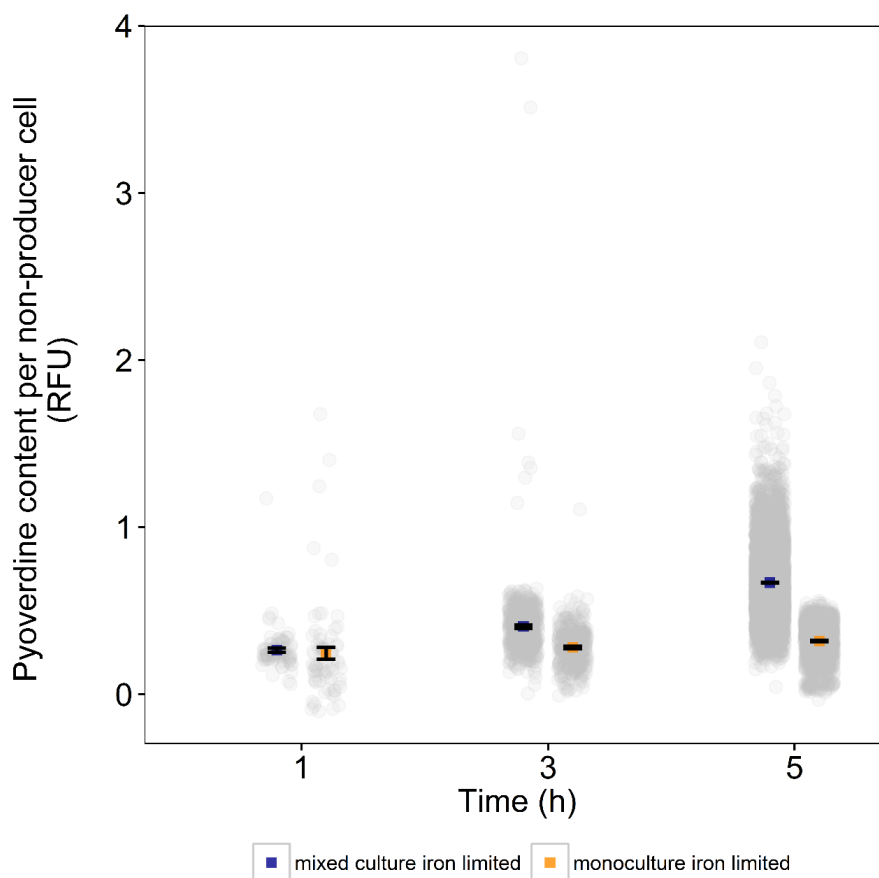
## **Data Archiving Statement**

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.hb8b5>

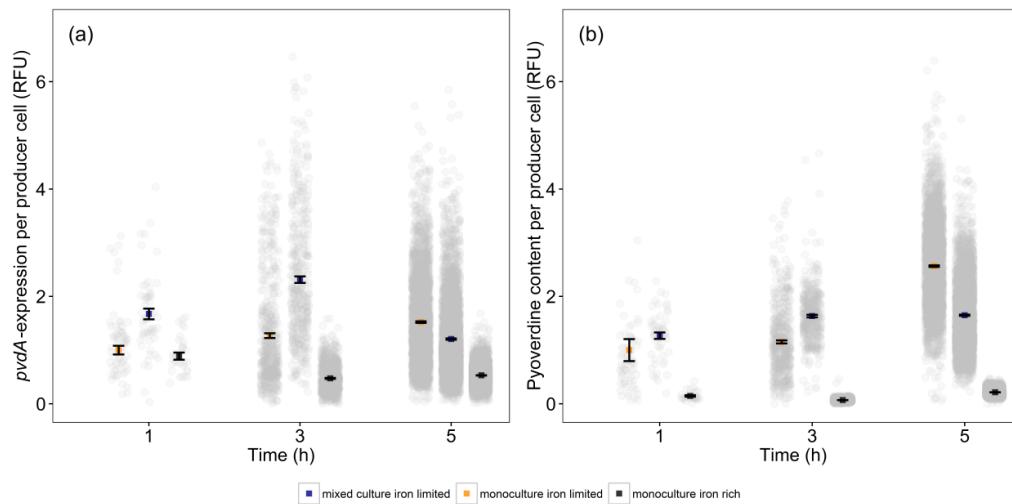
## **Competing interests**

None declared.

## 5.1 Supporting Material

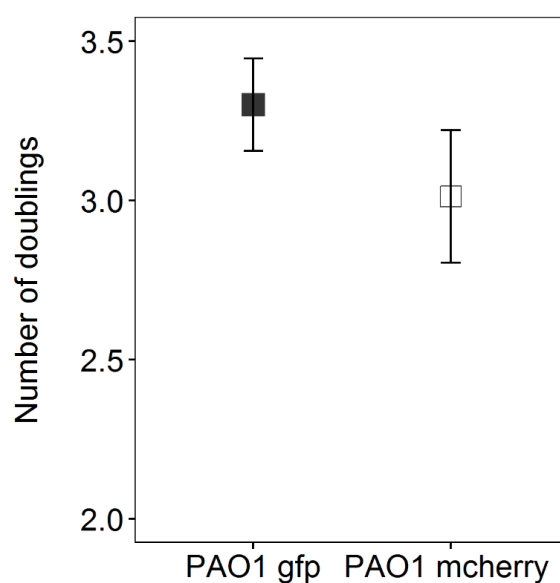


**Figure S1:** Pyoverdine is taken up by non-producing cells in a time-dependent manner, demonstrating pyoverdine sharing between physically separated, surface-attached micro-colonies. Time-course measures on natural pyoverdine fluorescence units (RFU) shows constant background fluorescence in non-producer cells grown in monocultures (orange squares), whereas pyoverdine fluorescence significantly increased in non-producer cells grown in mixed cultures with producers (blue squares). Mean relative fluorescence values  $\pm$  standard errors are scaled relative to producer monocultures after one hour of growth. Important to note is that only apo-pyoverdine (i.e. iron-free) is fluorescent, and therefore the measured fluorescence intensities represent a conservative measure of the actual pyoverdine content per cell. Furthermore, the fluorescence intensity in producer cells is always higher than in non-producer cells because it represents the sum of pyoverdine taken up from the environment and newly synthesized pyoverdine, whereas for non-producers, fluorescence represents pyoverdine uptake only.

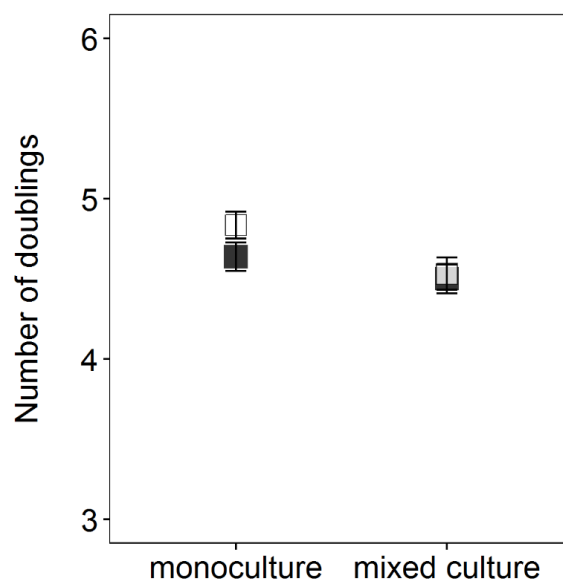


**Figure S2:** Producer cells adjust their pyoverdine investment level in response to changes in the social environment. **(a)** Time-course data show that *pvdA*, a gene encoding an enzyme involved in pyoverdine synthesis, is down-regulated in iron-rich media (grey squares), but up-regulated in iron-deplete media. Importantly, producers exhibited different *pvdA* expression patterns depending on whether they grew together with non-producers (blue squares) or as monoculture (orange squares). While producers showed increased gene expression in mixed compared to monoculture after one and three hours, the pattern flipped after five hours. **(b)** The same qualitative pattern was observed when measuring pyoverdine content per cell, as relative fluorescence units (RFU). Fluorescence values are scaled relative to the producer monocultures after one hour of growth. Important to note is that only apo-pyoverdine (i.e. iron-free) is fluorescent. Error bars indicate standard errors of the mean.

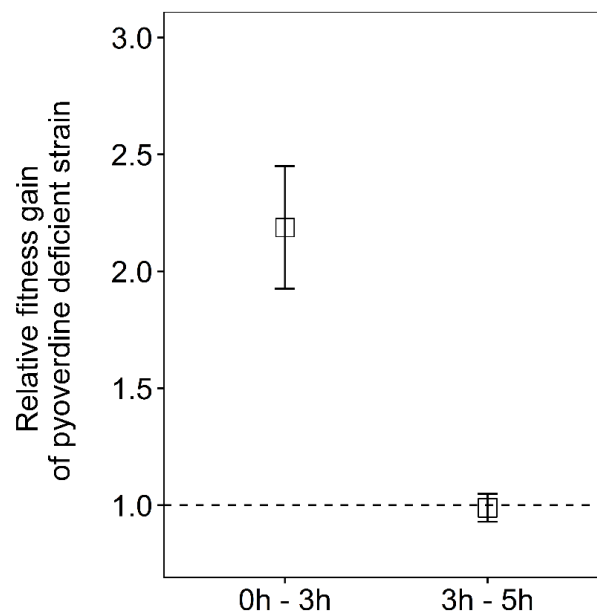




**Figure S3:** Growth did not differ between strains tagged with GFP or mCherry. We grew the wildtype PAO1 strain, either tagged with GFP or mCherry, on iron-limited agarose pads and calculated the number of doublings over 5 hours. Doubling numbers did not significantly differ between the two strains (t-test:  $t_{94} = 1.14$ ,  $p = 0.258$ ). Thus, we can be confident that growth differences observed in our experiments are due to biological and not tag effects. Symbols and error bars indicate means and standard errors of the mean, respectively.



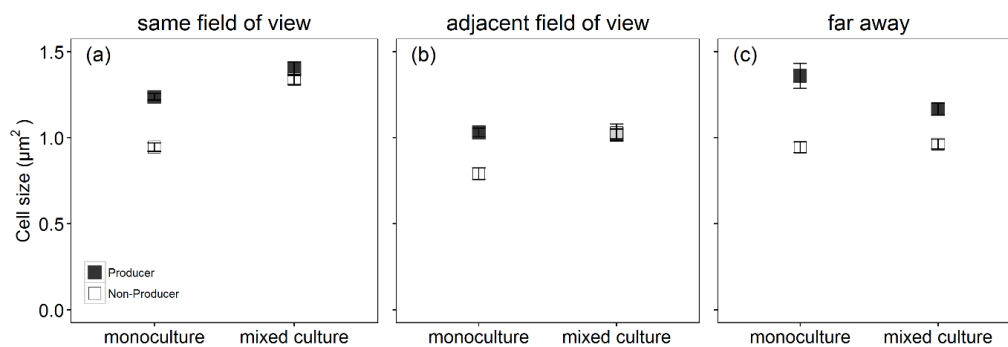
**Figure S4:** There are no growth differences between the pyoverdine-producing strain (filled squares) and the non-producing strain (open squares) on agarose pads supplemented with 200  $\mu\text{M}$   $\text{FeCl}_3$ . Growth of the two strains was neither different in monoculture (t-test:  $t_{78} = -1.61$ ,  $p = 0.11$ ) nor in mixed culture (t-test:  $t_{71} = -0.23$ ,  $p = 0.82$ ). These results are in line with the view that pyoverdine production is completely stalled when iron is plentiful (46), such that there is no more difference in the strains' phenotype. This also means that the fitness effects we observed in iron-depleted media (Figures 3 - 6) are attributable to pyoverdine-mediated social interactions. The number of doublings was calculated over a growth period of 3 hours. Symbols and error bars indicate means and standard errors of the mean, respectively.



**Figure S5:** Non-producers experience a significant relative fitness advantage during the first three hours of competition (zero to three hours; one sample t-test:  $t_{20} = 4.53$ ,  $p < 0.001$ ), but not during the later competition phase (three to five hours; one sample t-test:  $t_{41} = -0.18$ ,  $p = 0.85$ ). We used cell numbers to calculate strain frequencies at time point zero, three and five hours and to estimate the relative fitness of non-producers as  $v = [q_2(1 - q_1)]/[q_1(1 - q_2)]$ , where  $q_1$  and  $q_2$  are the initial and final frequencies of the non-producer (Ross-Gillespie et al. 2007)<sup>a</sup>. The dotted line represents the fitness equilibrium, where no strain has a relative fitness advantage over the other. Symbols and error bars indicate means and standard errors of the mean, respectively.

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<sup>a</sup> Ross-Gillespie A, Gardner A, Buckling A, West SA, Griffin AS. Frequency dependence and cooperation: theory and a test with bacteria. *Am Nat.* 2007;170(3):242–331. DOI: 10.1086/519860



**Figure S6:** Pyoverdine sharing affects cell size. While non-producer cells (open squares) were significantly smaller than producer cells (closed squares) in monocultures, non-producer cell size was restored to wildtype level in mixed cultures when the producer microcolony was (a) within the same field of view (average distance between cells  $36 \mu\text{m}$ ), (b) in an adjacent field of view (minimal distance  $\sim 100 \mu\text{m}$ ), but not when producers were far away (on opposite ends of the agarose pad) (c). Cell size was measured after three hours of growth. Error bars indicate the standard error of the mean.

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**Automated Segmentation and Single Cell Analysis for Bacterial Cells**

-Description of the workflow and a step-by-step protocol-

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**Authors:**

Michael Weigert and Rolf Kümmerli

## 1 General Information

This document presents a new workflow for automated segmentation of bacterial cells, and the subsequent analysis of single-cell features (e.g. cell size, relative fluorescence values). The described methods require the use of three freely available open source software packages. These are:

1. ilastik: <http://ilastik.org/> [1]
2. Fiji: <https://fiji.sc/> [2]
3. R: <https://www.r-project.org/> [4]

Ilastik is an interactive, machine learning based, supervised object classification and segmentation toolkit, which we use to automatically segment bacterial cells from microscopy images. Segmentation is the process of dividing an image into objects and background, a bottleneck in many of the current approaches for single cell image analysis. The advantage of ilastik is that the segmentation process does not require priors (e.g. information on cell shape), and can thus be used to analyze any type of objects. Furthermore, ilastik also works for low-resolution images. Ilastik involves a user supervised training process, during which the software is trained to reliably recognize the objects of interest. This process creates an "Object Prediction Map" that is used to identify objects in images that are not part of the training process. The training process is computationally intensive. We thus recommend to use of a computer with a multi-core CPU (ilastik supports hyper threading) and at least 16GB of RAM-memory. Alternatively, a computing center or cloud computing service could be used to speed up the training process. The training process, although time consuming, has to be carried out only once. Afterwards, the trained classifier is applicable to all experiments with the same type of objectives.

Segmentation is then followed by high throughput extraction of cell parameters in Fiji. For that purpose, we developed specific macro scripts (see Fiji\_scripts part1 - 5). We will explain the use of these scripts in detail in the "Walkthrough" section (4) below. The current version of the scripts are adapted to work with the attached example files. It might be necessary to adjust them to the user's specific needs.

In a final step, the extracted cell parameter data will be important into a R-based graphical interface called ShinyApp [5], which we specifically programmed for this workflow. This step is required because the information on cell parameters need to be connected to the descriptive variables (e.g. date, time, image\_ID, treatment, channel, etc.) of the experiment, which are at this stage only encrypted in the file title. The ShinyApp is a user friendly interface and does not require knowledge of R programming. Once this step is completed, a data set containing all analyzed images can be extracted as a spread-sheet in csv-format, which can then be fed into any standard statistical software package for in-depth analysis.

## 2 Methods Part I: Training of ilastik and Automated Segmentation

It is beyond the scope of this document to provide detailed instructions on how to use ilastik. We refer readers to the detailed instruction manuals available on <http://ilastik.org>. The documentations contain an in-depth descriptions of all the required steps (in ilastik), we briefly outline below.

### 2.1 Getting Started

In this step, we feed training pictures to ilastik and choose the starting conditions for the training phase. To begin this process, we choose the “Pixel Classification + Object Classification” workflow. Next, ilastik asks for training images, which should cover the full spectrum of variation observed within an experiment (e.g. variation in cell numbers, contrast, cell size, etc.). We typically perform segmentation based on phase contrast images, but it also works with bright field or fluorescence images. In the next applet, we then select features that will be used to classify pixels (e.g. Colors, Edges, Textures, etc.). It is advisable to start with a wide range (or even all) features, which can subsequently be reduced if necessary.

All steps below should be repeated for all training images until object recognition is satisfactory (*it might be necessary to go back and forth between the images, especially if cell numbers differ*). Now, we are ready to launch the training process.

### 2.2 Training Procedure

In the training process we supervise the object classification in ilastik. Based on this manual classification, ilastik will create a so-called “Object Prediction Map” that is later used for segmentation. *The training process should be performed in the “Off-mode”, since the “Live-Mode” is computationally very expensive. To do so, toggle the button “Live Update”. Training is in the “Off-mode” when red circles with a black crosses appear behind the label names.*

1. Create two labels, one for the background and one for the objects.
2. Mark the background and the objects on the training images with the respective labels (Figure 1A & 2A: red = background, green = object).
3. To control the result, toggle to “Live Update”. *This option will compute the “Object Prediction Map”. If the object identification requires further improvement, toggle back to the “Off-mode” and repeat step 2.*
4. Only applicable for low resolution/contrast images: To better distinguish the objects from the background we can only mark the part of the cell with the highest contrast (Figure 1 B), which is usually the center of the cell. This will improve object classification but also introduce a bias in cell size. This bias can be corrected at a later stage (step 3.2; Figure 1C).

### 2.3 Thresholding Images Based on Object Classification

Step 2.2 creates an “Object Prediction Map” that is now used to segment the image (result of segmentation: Figure 2B). It might be necessary to switch back and forth multiple times between the training and the thresholding mode to improve segmentation.

1. Set “Input Channel” to the label you chose for the objects (e.g. Label 2 → Green → “Input Channel” = 1).
2. We set “Sigma” to small values (e.g. 0.1). Sigma uses a Gaussian to smooth the “Object Prediction Map”, which is not necessarily needed. *For details, please refer to the ilastik-documentation.*
3. We choose an intermediate “Threshold” (e.g. 0.5), in order to reliably segment the different objects from one another. *The threshold value allows us to change the size of the objects, which are recognized as objects. Higher values indicate smaller objects an vice versa.*

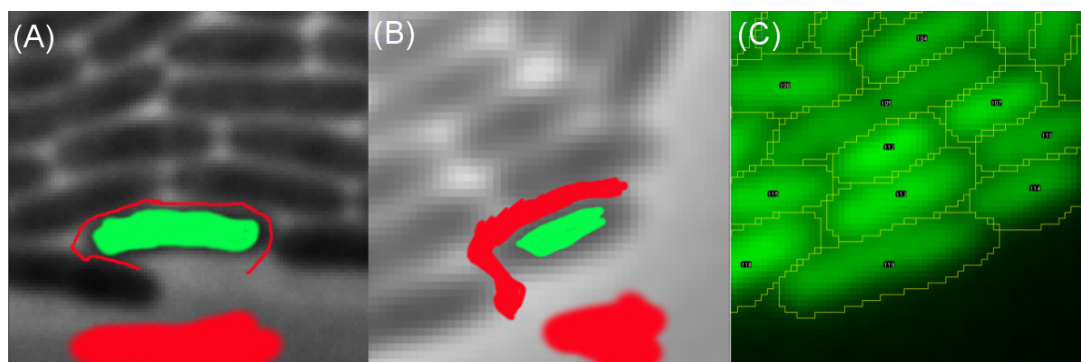


Figure 1: **(A)** High resolution/contrast images. In the training process 2.2 we can mark the whole cell with the "Object Label" (green) since resolution is high enough to separate cells. **(B)** Low resolution/contrast. Only the center of the cell with high contrast level is marked with the "Object Label" (green). Resolution is not high enough to reliably separate cells, therefore we need to clearly separate the cell with the "background label" (thick red line). By only marking areas with high contrast level, we introduce a bias in cell size, which can be corrected later. **(C)** Here we show a gfp-fluorescence image, where the bias, introduced in (B), is already corrected (step 3.2). Outlines of the cells now perfectly match the boarder of the cells. The possibility to correct for size bias allows us to reliably segment images, even if the used camera resolution is poor (e.g. high sensitivity cameras)

4. We choose an appropriate "Size Range" (e.g. 10-1000000). This parameter is useful to exclude non-biological objects such as dust particles.
5. By pressing "Apply", the threshold-settings are applied to the image (Figure 2B). Every object should now be appear in a different color.
6. If the segmentation is satisfactorily the move to step 2.4. If an error is spotted, try to change the threshold or go back to training mode.

## 2.4 From Segmentation to Objects

This step ("Object Feature Selection") calculates the features of objects (Figure 2C). *For details see ilastik documentation.*

1. Select "All features". *The amount of calculated features can be reduced any stage.*
2. Switch to the applet "Object Classification".
3. Create two labels, choose the "Object Label" (green) and click on one cell to mark it.
4. Toggle "Live Preview" to identify all objects.

## 2.5 Export Objects

These objects (from step 2.4) can now be exported (Figure 2D), as a binary black and white image.

1. Switch to the applet "Object Information Export"



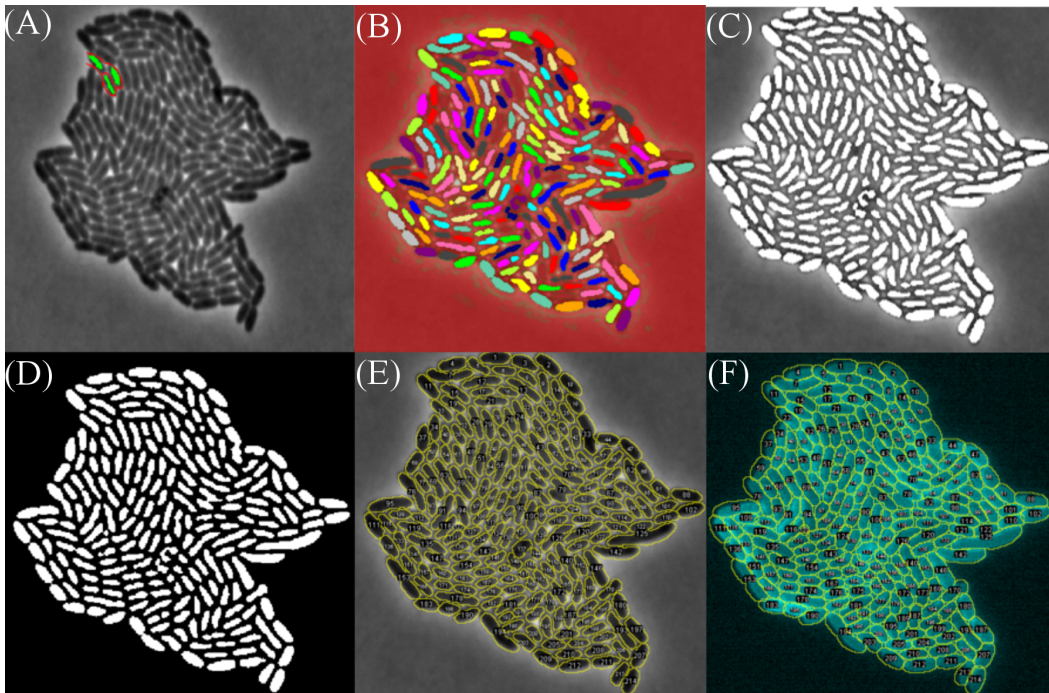


Figure 2: (A) Objects (green) and background (red) were marked with the corresponding label (step 2.2 in ilastik). (B) Visualization of segmentation, based on the training procedure (step 2.3 in ilastik). Every cell will appear with an individual color. If results are not satisfactory, either the threshold-value has to be changed or cells have to be manually separated in the training step (2.2) (C) Based on training and segmentation, ilastik predicts objects (step 2.4). (D) Predictions are exported as a black and white image (here opened in Fiji; step 2.5). (E) This segmentation image can be used to create regions of interest (ROIs; yellow outlines) in Fiji. (F) ROIs can then be used to measure cell features in different images e.g. RFU in different channels.

2. Open export configuration by clicking on "Choose Export Images Settings..."
3. "Change Output File Info" to "png" and save by clicking on "OK".
4. Press "Export" to export one image. *The exported Image will be saved in the folder, where the example images are located. Ilastik will extend the original filename by "\_Object Prediction".*
5. As a quality control, we can now load a representative image into Fiji. If necessary adjust contrast levels by applying "Auto" (IMAGE→ADJUST→THRESHOLD).
6. Check whether segmentation was successful (image should look like figure 2 D).

### 2.6 Automated Segmentation

If the "Object Prediction" is successful and satisfactory, we can use the "Batch Input"-function to automatically apply the segmentation to all images in a folder.

### 3 Methods Part II: Extracting Parameters from Single Cells

Now we switch to Fiji. Fiji is an image processing package to facilitate scientific image analysis, based in ImageJ [3]. It is freely available under <https://fiji.sc/>. A step-by-step walkthrough for the following protocol can be found in section 4 (including example images).

Each of the following sections includes a short description of the implemented process, the Fiji-commands needed to carry out the process on a single image, and information on how to automatically apply the process to all images.

#### 3.1 Thresholding Images in Fiji and Creating Uncorrected ROIS

In this step we will use the binary images received from ilastik to create regions of interests (ROIs), which can later be used to extract cell parameters (e.g. length, RFU, etc.) from any type of image (e.g. fluorescent images). To create ROIs we first have to threshold the binary images in Fiji. *Thresholding: Choosing a cutoff value, so that every pixel below or above the threshold will be considered as background or object, respectively.*

1. Open a binary image in Fiji (Figure 2D).
2. Threshold images: **IMAGE**→**ADJUST**→**THRESHOLD**; *Setting: Upper slider 1, Lower slider 2, Rest: Default;B&W*
3. Now we can create ROIs with "Analyze Particles" (Figure 2E). **ANALYZE**→**ANALYZE PARTICLES**; *Settings: CLEAR RESULTS, ADD TO MANAGER, EXCLUDE ON EDGES. With the "Size" and "Circularity" argument we can exclude objects which are not cells.*
4. The generated ROIs can now be saved by selecting **MORE**→**SAVE** in the ROI Manager. *ROIs will be saved in a .zip-container.*

Script for batch process: PART1\_CREATING\_THRESHOLD\_IMAGES

Remarks for skript usage: It can be desirable to change size and circularity of objects for batch processing. In this case, the **size=0-Infinity** and **circularity=0.00-1.00** arguments can be changed in the following command-line: `run("Analyze Particles...", "size=0-Infinity circularity=0.00-1.00 exclude clear add")`.

#### 3.2 Correcting for Segmentation Biases and x,y-Position of ROIs

In this step we can correct for segmentation biases we might have introduced at the segmentation step (see step 2.2/4). Moreover, we can also correct for drift in the x, y position of the ROIs, which can occur during filter cube change. Once we have defined the correction values they can automatically be applied to all images using the following two scripts. Script:

- PART2\_MANUALLY\_SHIFT ROI
- PART3\_MANUALLY\_SIZE\_ADJUST

Remarks for script usage: The script applies the same corrections to all images. Please verify whether corrections generate reliable ROIs.

### Creating Corrected ROIs (only for batch processing)

We wrote a script that allows automated adjusting of all ROIs:

PART4\_CREATING ADJUSTED ROIs

### 3.3 Extracting Cell Parameters and RFU

Here we can extract information from the ROIs we have created above. First we have to set the parameters we want to measure (e.g. fluorescence intensity, cell size, cell shape). A full list of parameters that Fiji can measure (incl. description) can be found here: <https://imagej.nih.gov/ij/docs/menus/analyze.html>.

1. Choose the parameters you want to measure **ANALYZE**→**SET MEASUREMENTS**; *Make sure that the option for "Display Label" is checked.*
2. Open an image and the corresponding ROI. In the ROI-Manager check the option: "Show All".
3. Measure parameters with: **ANALYZE**→**MEASURE**.
4. Copy the results into an Excel-file and save as .csv (separated by comma, semicolon or tab).

We wrote a script that allows automated batch processing of all images: PART5\_MEASURING

#### Remark for skript usage:

Image names and names of the ROIs have to correspond exactly, for the automated script to work. We have included an option in the script that allows to change the file name (of the file loaded), if names of images and ROIs do not correspond (e.g. CH1 and CH2; see section 4 for details).

### 3.4 Assigning Factor Levels to Data

From the generated file, we have extract information that are encoded in the factor name (see table 1 and table 2). We can do this in R-based ShinyApps (this step is simple to execute and will be briefly explained in 4).

Name	Area	RFU
24122016_TreatmentA_Factor1_..._CH1.tif	1	1000

Table 1: The exported Excel-file from Fiji lists important image information (e.g. time, treatment, factor level, channel) in a concatenated form under the label "Name".

Date	Treatment	Factor	...	Chanel	Area	RFU
24122016	TreatmentA	Factor1	...	CH1	1	1000

Table 2: The ShinyApp allows to split up the relevant information into different columns for subsequent analysis.

## 4 Walkthrough with Example Dataset

This walkthrough explains the application of the Fiji scripts using representative example images. It starts with the binary images received from ilastik.

### Comments before we can start the procedure:

- Name of example image received from ilastik:  
3h\_control\_Image002\_CH1\_Object Predictions.png
- "\_Object Predictions.png" is added to the original filename by ilastik and is essential for the script to work. It is possible to remove/change this extra label by changing change the "Object Predictions.png"-argument in script: `PART1_CREATING_THRESHOLD_IMAGES; filename=replace(title,"Object Predictions.png","");`

### Starting the Procedure

1. Start Fiji and open script `part1_creating_threshold_images` and run it. Follow the instructions.
2. Go into the folder `EXAMPLE DATA\GFP` and load one of the gfp-images.
3. Load the corresponding ROI.zip-file.
4. Select the gfp image, and in the ROI-Manager check the option "Show all".
5. Zoom in and check alignment (size, and x/y position). To correct alignment, run script `PART2_MANUALLY_SHIFT ROI` and/or `PART3_MANUALLY_SIZE_ADJUST`. To undo the changes, "re-load" the ROIs or rerun the script with inverted signs (e.g.  $- \rightarrow +$ ) using the same values. Please remember the implemented correction values, as they will be needed in the next step. *Correction factors are sensitive to scale, it might be necessary to set the scale in your image.* (<http://imagej.net/SpatialCalibration>).
6. To create corrected ROIs, open and run script `PART4_CREATING_ADJUSTED_ROIs` and enter the correction values from the previous step.
7. To measure cell features (e.g. size, RFU,...), follow the instructions from step 3.3/1 first ("Set measurement").
8. To extract object parameters of interest, open and run script `part5_measuring`. *Please note, that ilastik did the segmentation based on phase contrast images, which corresponds to channel name "CH1". GFP-images were, however, recorded in channel "CH2". If we want to measure RFU in GFP-images we thus have to change the name of the file, since the script requires the names to correspond exactly. We have implemented an automated renaming option in the script. When running the script, it first asks for the name of the channel used for segmentation (here "CH1") and then the name of the channel to be analyzed (here "CH2"). This option will simply adjust the file names while being processed by Fiji, and it will not change the actual filename in the folder.*
9. Copy results from the output table into an excel file and save as .csv (with column names) in the folder.
  - (a) Download R or use a portable version.

## 5.1 Supporting Material

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- (b) Start R and run our script `SPLIT_DATA_GUI.R`. In a first step, the script First it will automatically download and install all the necessary packages<sup>1</sup>
- (c) A new tab/window in your browser will open with the graphical ShinyApp interface.
- (d) Follow the instructions on the left-hand side of the interface, from top to bottom. In a first step, upload the csv-file, then you can assign, split and rename factors. Finally, the modified table can be exported as csv-file and used for in-depth statistical analysis and plotting.

## References

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- [5] Chang W. et al., *Shiny: Web Application Framework for R*, (2017)

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<sup>1</sup>Once the packages are installed, following two lines are not longer needed: `install.packages("shiny",repos='http://cran.us.r-project.org')` and `install.packages("splitstackshape",repos='http://cran.us.r-project.org')`

## CHAPTER 6

### Concluding Discussion

*Nothing in Biology Makes Sense Except in the Light of  
Evolution*

Theodosius Dobzhansky 1973

A word cloud of biological and medical terms. The most prominent words are 'Virulence' (in large red letters), 'Resistance' (in large red letters), 'Anti-virulence' (in large blue letters), and 'Infection' (in large blue letters). Other visible words include 'Pseudomonas', 'Antibiotic', 'Treatment', 'Efflux', 'Therapy', 'Siderophore', 'Acute', 'Pathogen', 'disease', 'Drug', 'Pyoverdine', 'Target', 'Chronic', 'Gallium', 'Clear', 'Host', 'Quorum', and 'Quorum'.

## 6.1 Overview

Cooperation is a ubiquitous feature in the bacterial world and especially virulence is often facilitated by cooperative interactions, as in *P. aeruginosa*, where cooperation is crucial to successfully colonize a hosts. Often this is facilitated by secreted, publicly shared virulence factors [23, 94, 95, 99, 100].

In chapter 3, we used an approach that targets the secreted and publicly shared virulence factor pyoverdine to reduced pathogenic growth and consequently virulence in an insect model. We achieved this by exposing *P. aeruginosa* to gallium (at concentrations that are not harmful to the host), which binds preferentially to its siderophore, pyoverdine, rendering it useless. Moreover, we subjected *P. aeruginosa* to gallium or different antibiotics (and a combination of those) over a period of two weeks. While bacteria rapidly gained resistance to antibiotics, gallium retained its efficacy, indicating that bacteria indeed have a hard time to become resistant against gallium. These results support our hypothesis that the anti-virulence agent gallium can be an evolution proof treatment (that is a treatment to which bacteria cannot easily develop resistance).

In chapter 3, we showed that gallium-induced pyoverdine quenching (*in-vitro*) is accompanied by an upregulation of pyoverdine production as a compensatory mechanism. This might have dramatic consequences for the regulatory network of the pathogen and can lead to overproduction of other, regulatorily linked, virulence factors, which would be detrimental for gallium as a treatment. Therefore, in chapter 4, we investigated the effect of the manipulation of virulence factor availability (by gallium) on virulence, the regulatory network and the host response. We found that virulence factor availability was overall predictive for virulence and that manipulating virulence factor availability feeds back the regulatory network of the pathogen. Furthermore, the immune

response of the host was elevated, when confronted with the pathogen and with increasing virulence factor availability. However, we did not observe increased virulence, when gallium was present. Nevertheless, these insights show that we need a close understanding of how any given anti-virulence agent works in order to design effective and evolutionary robust anti-virulence treatments.

Our argument of evolutionary robustness is based on the assumption that we targeted a shareable virulence factor. Many studies have shown the shareability of pyoverdine in liquid cultures with millions of cells and that pyoverdine is a virulence factor [22, 23, 37, 94–100]. However, we have little knowledge about the shareability of pyoverdine in a less artificial setup, where diffusion of pyoverdine might be limited, due to increased viscosity (e.g. tissue or biofilms). Therefore, we conducted experiments with surface attached bacteria and established the physical limits of pyoverdine sharing (chapter 5). We found that pyoverdine is collectively shared, even when its diffusion is severely limited and cell numbers are low, conditions *P. aeruginosa* could for example experience during colonization of a host or within biofilms. Hence, at this stage *P. aeruginosa* would be most vulnerable to therapies, which aim to inhibit its siderophore.

In the following sections, I will discuss the key insight of each chapter in a wider context.

## 6.2 Gallium Therapy and Resistance Evolution

In chapter 3 we proposed gallium as a potentially evolution proof anti-virulence treatment that reduced pathogenicity of *P. aeruginosa* in an insect model and in mice [163]. Gallium binds irreversibly to pyoverdine, making it unavailable as an iron delivery system [162]. It is predicted, that gallium should not induce resistance, since it is targeting a secreted and collectively shared virulence fac-



tor [96, 142] and section 2.5. Thus, routes to resistance are limited and in case resistance arises, it is selected against. And indeed, we did not find any signs of resistance during an experimental evolution, where bacteria were subjected to gallium over a period of two weeks, whereas, bacteria, exposed to antibiotics, developed resistance rapidly. But what does this mean for gallium as a potential drug? Will there be no resistance whatsoever?

### 6.2.1 What is Resistance?

Resistance to a treatment is often defined as the recovery of growth [123]. Since antibiotics kill bacteria (bactericidal) or dramatically reduce proliferation (bacteriostatic), growth is a good indicator for resistance. However, resistance to an anti-virulence treatment could manifest in at least three ways: (i) recovery of growth, (ii) restoration of virulence factor production and (iii) detection of bypassing mechanisms.

Anti-virulence agents are not primarily designed to hinder pathogenic growth. They disarm pathogens by targeting their virulence factors and since there is a profound disconnect between growth and virulence factor production [97, 144], recovery of growth alone might be not enough to define resistance. A pathogen may recover its growth in an infection (treated with an anti-virulence agent), but since virulence factors are quenched, it could do so in a benign, non-virulent state, where it is causing no (or less) damage to the host. For anti-virulence treatments, we therefore have to include full restoration of virulence (by e.g. bypassing the virulence factor) into the definition of resistance.

### 6.2.2 Assessing Resistance to an Anti-Virulence Treatment

Assessing resistance to a drug can be carried out within the host (*in-vivo*) or in a laboratory medium (*in-vitro*). Both approaches have their advantages and disadvantages. The identification of virulence factors often involves a screen in laboratory medium for non-essential genes that are predictive for a model-host

system. Experiments in laboratory media are easy to perform, but simultaneously are very artificial, as the virulence factor might not even been deployed (e.g. some virulence factors might require cues from a host for their expression). Such a setup differs greatly to conditions where selection for resistance happens, inside the host [201]. Thus, resistance development to anti-virulence drugs should be assessed *in-vivo*, where the inhibited virulence factors (among other) are detrimental for the pathogen. But evolution experiments within a host are difficult to carry out and are naturally subjected to increased variation (introduced by the differences between hosts).

In our experiments (chapter 3), we performed the test for resistance in CAA (casamino acids; our standard laboratory medium for iron limited experiments), but this experiments not necessarily reflect what is happening in the host. Therefore, we aim to investigate the potential of *P. aeruginosa* to develop resistance to gallium in an *ex-vivo* growth medium, namely human serum<sup>1</sup> (HS). Human serum is a complex, protein rich medium with iron binding factors (e.g. transferrin) [202]. It was shown that, in addition to proteases, *P. aeruginosa* also requires pyoverdine to grow [202]. Moreover, we observed that QS deficient strains ( $\Delta lasR$ ) are unable to grow in HS, whereas a RhIR-deficient strain can proliferate, indicating that LasR-controlled proteases are essential in HS (Rezzoagli C., Wilson D., Wyder S., Weigert M. and Kümmerli R., 2017, in preparation). When predigested with proteinase K, growth of *P. aeruginosa* was elevated, irrespective of pyoverdine and protease production [202]. This indicates that HS promotes the production of proteases and possibly other virulence factors to facilitate growth. Thus, HS combines advantages of *in-vivo* and

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<sup>1</sup>Human Serum: Blood is collected and separated into cells and plasma. The plasma is separated from the cells, and calcium is added to activate the clotting cascade of the plasma. Subsequently the mixture is centrifuged, and the liquid product remaining is the serum. From production sheet by Sigma Aldrich.

*in-vitro* approaches to assess possible resistance mechanisms. HS could allow more routes to resistance (e.g. virulence factors, which are not advantageous in laboratory media, become beneficial in HS and can be used to bypass the quenched virulence factor) than standard laboratory media and thus is a better environment to test for resistance.

## 6.3 Consequences of Manipulation of Virulence Factor Availability

In chapter 3 we established that the addition of gallium upregulates pyoverdine production as compensatory mechanism. This might lead to increased expression of Protease IV and ETA via the genetic link described previously (section 2.3.3). Therefore, instead of reducing virulence, gallium would increase it. Enhanced pyoverdine secretion could also affect the host as it might act as a cue for the host to upregulate a specific immune response. An elevated immune response could easily lead to an arms race between the host and the pathogen and thus can cause unpredictable treatment outcomes. Therefore, we performed a detailed analysis of the effects of anti-virulence treatments on the pathogen, its regulatory network and the host (chapter 4).

We found that virulence factor availability did not correlate monotonously with either pathogenic growth *in-vivo*, nor with virulence. We did not observe a strong response of ETA- or Protease IV-expression to gallium in our experiments. However, it is unclear if addition of gallium did not induce expression changes of these virulence factors, or if their expression is limited due to the use of laboratory media (non-pathogenic environment). Assessing such regulatory changes *in-vivo* would be more informative, but it is difficult to recover sufficient amounts of bacterial RNA from the host for later analysis. Potentially, we could perform this experiment in HS, but it would still be contaminated with

human RNA (from the donor). However, reduced virulence in gallium treated larvae indicate that, if such upregulation is happening, its effect is negligible [96, 97].

Moreover, we found that the host is reacting to the manipulation of the virulence factor pyoverdine. This implies that virulence factor availability is involved in triggering host response. In humans this could be facilitated by siderocalin, a protein which is part of the innate immune response and able to bind siderophores to prevent bacterial iron uptake [203]. This could slow down pathogenic growth, which would give the immune system an edge and more time to fight back the infection.

Together, these results show that we are in the need of a close understanding of anti-virulence treatments and their effect on the bacteria and the host in order to design effective and evolutionary robust treatments.

## 6.4 Perspectives

What are the perspectives of anti-virulence treatments and their potential applications in infections? In chapter 3 and 4 we proposed gallium as an resistance-proof agent to fight *P. aeruginosa* infections. Our studies support the concept of evolution proof anti-virulence treatments. But can, for example, gallium be used as a drug to control infections?

### 6.4.1 Gallium as a Drug?

Gallium is not a new therapeutic agent. In its commercial, FDA-approved form Ganite (Genta), it is used as a anti-cancer drug, relying on elevated uptake rates of cancer cells, inhibiting iron-dependent metabolic functions [204].

In March 2016 the university of Washington started a phase 2 clinical study with gallium in cystic fibrosis patients (ClinicalTrials.gov Identifier: NCT02354859), which is expected to finish in April 2017. The primary goal of this study is to

improve pulmonary function (by 5% or more) and as a secondary outcome, to reduce abundance of *P. aeruginosa* in CF-lungs of participants. However, the outcome of the study might not answer the question whether gallium helps to reduce the virulence of *P. aeruginosa* in the way we proposed here, as an anti-virulence drug. Patients eligible for the trial must have a chronic CF-associated infection. But it is known that *P. aeruginosa* often loses its ability to produce pyoverdine in chronic CF-infections [98, 101, 102]. As described in chapter 3, gallium can only work as an anti-virulence agent at intermediate concentrations, where it inhibits pyoverdine, but diffusion driven gallium-uptake across the cell membrane is presumably not taking place [96]. However if no siderophores are present, gallium can only work by its antibacterial effect (at high concentrations), when it increasingly diffuses across the cell membrane, and displays off-target effects, e.g. disrupting iron dependent processes within the cell. Consequently, it becomes vulnerable to resistance mechanism, similar to the ones known from antibiotics (e.g. efflux pumps). This shows that we have to evaluate the correct dose at which an anti-virulence drug has to be administered, since too little of the drug may have no effect, but too much of it could change it from an anti-virulence agent to a treatment with off-target effects.

Following this line of argumentation, treating chronic CF-infections with gallium might not be an anti-virulence treatment. Its administration is happening too late and gallium cannot effectively work as an anti-virulence treatment (since bacteria might no longer produce pyoverdine). This, however, raises the question, when administration of gallium would be most effective?

### **6.4.2 When Would Administration of Gallium be Most Effective?**

Based on the insights we gained from our experiments in chapter 5, we know that pyoverdine cooperation is already taking place when cell numbers are very low and when the viscosity of the environment was elevated. We established

that pyoverdine diffusion and sharing is happening shortly after incubation, facilitating the growth of pyoverdine non-producing strains, without the need of cell-to-cell contact as previously claimed by Julou et al. [205]. Pyoverdine non-producer even benefited from pyoverdine producing strains when distance between the communities was greater than 60  $\mu\text{m}$ . *P. aeruginosa* could experience such conditions in early stages of infection in biofilms or within soft tissue.

By administering gallium, *P. aeruginosa* would suddenly be deprived of a virulence factor that is essential for colonization. The pathogenic community would consist of phenotypic pyoverdine non-producers that display reduced pathogenicity and might not be able to efficiently colonize the host [96, 97, 163]. Moreover, our results would imply that any individual that resumes cooperation (e.g. by becoming resistant to gallium) will be immediately exploited by the surrounding phenotypic non-producers, irrespective of the viscosity of the environment. Moreover, similar to our single cell experiments, resistant pyoverdine-producer would upregulate investment into pyoverdine production (as a compensatory mechanism for exploitation) at the cost of proliferation and eventually will get out-competed by the (phenotypic) non-producer.

The results imply that *P. aeruginosa* would be most vulnerable to gallium-mediated pyoverdine quenching when cell numbers are very low, or even as a preemptive treatment.

### 6.4.3 The Future of Anti-Virulence Treatments

Anti-virulence treatments can help to manage infections of multi-resistant pathogens, as they have many advantages: (i) they can reduce virulence of pathogens, (ii) can be designed to be resistant-proof and (iii) by their innate complexity, they can be very specific to a pathogen. Though, being a step forward in the right direction, anti-virulence treatments also come with drawbacks. Finding such drugs and evaluating all aspects of their mode of action and impact on the host and the pathogen (as discussed in this thesis) is difficult. Furthermore,

anti-virulence treatments may not reach the efficiency of antibiotics. But this is not necessarily what they are supposed to do, as they can be part of a combination therapy. Anti-virulence treatments can, for example, help to break up biofilms, making the pathogen more accessible for clearance with conventional antibiotics [161]. It has been shown that multi-resistant *P. aeruginosa*-strains, treated with a quorum quenching lactonase (the lactonase AiiA<sup>2</sup>), shows higher susceptibilities to gentamicin and ciprofloxacin, compared to a lactonase-free treatment [206].

As established earlier, anti-virulence treatments reduce the virulence of a pathogen and also can slow down their growth. This could give the immune system an edge and the chance to mount an appropriate immune response to fight back. Anti-virulence drugs are no weapons, as powerful as antibiotics were, when they entered the market in the 1940s, but they can help to manage infections, in cases where these "powerful" weapons fail.

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<sup>2</sup>AiiA is an enzyme produced by *Bacillus thuringiensis* to degrade AHLs [165].

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## **Appendix**

### **A.1 Illustration Directory**

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### **A.3 Acknowledgments**

First of all, I want to express my sincere gratitude to Prof. R. Kümmerli, for giving me the opportunity to be part of his research group and his support during my PhD-time. Thank you for all the help, endless discussion, support, scientific input. With your help, I was able to become the scientist, I am today.

I also would like to sincerely thank Prof K. Jung and H. Jung for giving me the opportunity to peruse my academic career by doing an external PhD and the LMU. Without your support, I would not have been able to do a PhD. Thank you, for all your time, help and support during the last years.

Next I would like to thank my committee, which agreed to read my thesis, without knowing me. Special thanks goes to Prof. Kai Papenfort for being my second examiner.

I would like to thank my group for endless scientific and non-scientific discussions, help in lab and for experiments. Thank you for providing such a nice working atmosphere.

Special thanks goes to Jakob, Kathrin and all the people who read this thesis and helped me to improve it.

I would like to thank the German Academic Exchange Service (DAAD) for financially supporting my stay in Switzerland.

Last but not least I'd like to thank Anne, my friends and my family. Without your support, I would not be, where I am today.

## A.4 Curriculum Vitae

# MICHAEL WEIGERT

### EDUCATION

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- 10/2013- External PhD-student at the Ludwig-Maximilians University Munich
- 04/2012 Diploma from the University for Applied Sciences Weihenstephan-Triesdorf
- 2012 Diploma thesis in the research group of Prof. Kümmerli at EAWAG, Dübendorf  
Topic: The impact of Durability of Siderophores on Virulence; In-vivo virulence tests using larvae of the Greater Waxmoth *Galleria mellonella*
- 10/2007- Study of Environmental Engineering at the University for Applied Sciences Weihenstephan-Triesdorf
- 04/2012 Applied Sciences Weihenstephan-Triesdorf
- 2007 University Entry Diploma (Abitur)

### PUBLICATIONS

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**M. Weigert**, R. Kümmerli. The physical boundaries of public goods cooperation between surface-attached bacterial cells. Proceedings B, 2017 (in press).

Ross-Gillespie, A.\* , **M. Weigert\***, S. P. Brown, R. Kümmerli. 2014. Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment. Evolution, Medicine, and Public Health 2014: 18-29.

**Weigert, M.**, A. Ross-Gillespie, A. Leinweber, G. Pessi, S. P. Brown, R. Kümmerli. 2017. Manipulating virulence factor availability can have complex consequences for infections. Evolutionary Applications 2017. 10: 91-101.

\* These authors contributed equally

### GRANTS

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- DAAD Jahresstipendium für Doktoranden
- University of Zurich, Department for Microbial and Plant Biology, Travel Grand for Conferences

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ACADEMIC ACTIVITIES

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- 2012 Workshop on Inclusive Fitness Theory, EPFL Lausanne, Switzerland
- 2013 Pseudomonas Conference, Lausanne, Switzerland, Poster
- 2014 15th International Symposium on Microbial Ecology, Seoul, South Korea, Poster
- 2015 International Conference for Evolutionary Medicine, Zürich, Talk
- 2015 European Society for Evolutionary Biology, Lausanne, Switzerland, Poster
- 2016 American Society for Microbiology, Boston, USA, Poster
- 2016 European Students' Conference, Charité Berlin, Germany, Invited Talk

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