How nutrients shape antibiotic sensitivity of Pseudomonas aeruginosa: food for thought

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Food for thought

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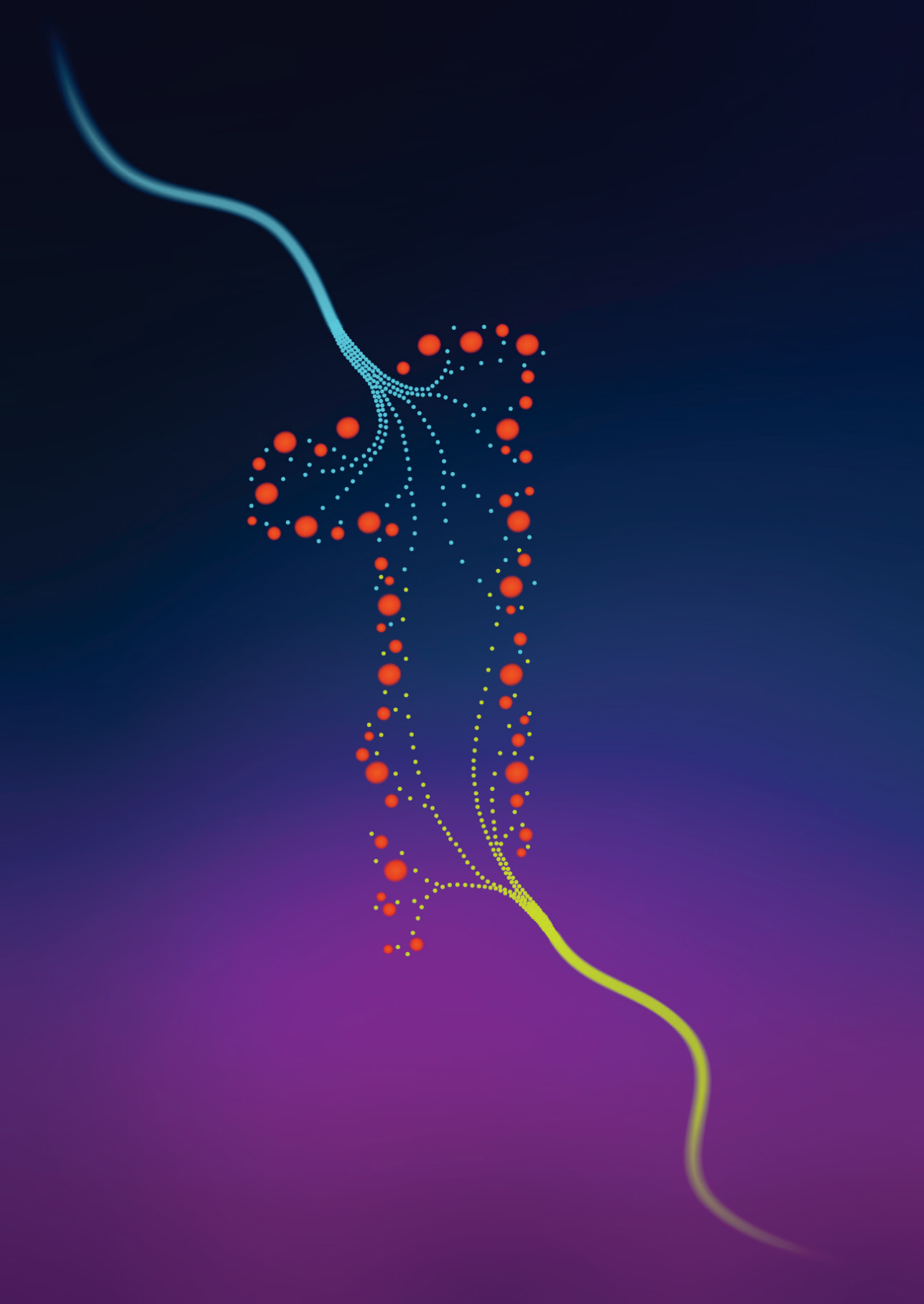

Section I

Introduction

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 1

General introduction and scope

1.1. Introduction

Pseudomonas aeruginosa: a high-risk pathogen

Pseudomonas aeruginosa is a versatile, opportunistic environmental pathogen responsible for a wide range of severe infections[1]. In 2017, the World Health Organization classified *P. aeruginosa* as a top-priority pathogen due to its severe threat to vulnerable patient populations[2]. Infections caused by *P. aeruginosa* are typically associated with the respiratory tract, wounds, and bloodstream, as well as catheters and medical implants[3]. Effective treatment of *P. aeruginosa* infections can be challenging due to high rates of multidrug resistance that reduces the effectiveness of many commonly used antibiotics, thereby limiting therapeutic options[4].

Eradicating *P. aeruginosa* is particularly challenging in respiratory tract infections among cystic fibrosis (CF) patients, where the pathogen frequently establishes chronic infections despite extensive antibiotic therapy[5,6]. The CF respiratory tract is characterized by thick mucus that impairs pathogen clearance, making control of bacterial load and infection-induced lung damage reliant on long-term suppressive therapy with multiple antibiotics[7]. This prolonged exposure to antibiotics promotes the development of antibiotic resistance[8,9], often leaving lung transplantation the only viable option for effectively clearing the infection[10]. Enhancing our understanding of P. aeruginosa pathogenesis and response to antibiotics in CF patients has the potential not only to improve clinical outcomes, but also to address the growing challenge of treating infections caused by multidrug-resistant P. aeruginosa strains.

Bacterial resilience is more than antibiotic resistance

Antibiotic resistance is a major global health threat, contributing to millions of deaths annually and representing the leading cause of antibiotic treatment failure[11]. Antibiotic resistance refers to the ability of bacteria to grow in the presence of antibiotic concentrations that would normally lead to growth inhibition or kill[12]. Commonly observed resistance mechanisms include mechanisms that lead to reduced antibiotic uptake, the modification of antibiotic targets, enzymatic inactivation of antibiotics, and the activation of efflux pumps which remove antibiotics from the cell[13]. Stable antibiotic resistance can arise

through spontaneous mutations in the bacterial genome or by acquiring mobile genetic elements through horizontal gene transfer. In addition to these genetic, stable resistance mechanisms, pathogens can also employ diverse transient mechanisms that allow bacteria to temporarily tolerate exposure to antibiotics[12]. These transient mechanisms vary by bacterial species and antibiotic class, involving phenotypic adaptations such as entering a dormant state[14], or genetic changes such as unstable gene amplifications[15]. The ability to transiently withstand antibiotic exposure is believed to facilitate the development of antibiotic resistance by providing additional opportunities for stable genetic traits to evolve and become fixed within bacterial populations[15–17]. This is particularly evident during the prolonged antibiotic treatments required to manage *P. aeruginosa* infections in the respiratory tract of CF patients. Consequently, there is a need to better understand how the distinct CF lung environment contributes to altered antibiotic sensitivity and the emergence of antibiotic resistance.

P. aeruginosa adaptation to the cystic fibrosis environment

The CF lung environment is characterized by thick, dehydrated mucus resulting from dysregulated electrolyte transport. This leads to limited oxygen penetration, impaired mucus clearance, and relatively high nutrient availability[18]. *P. aeruginosa* thrives in this challenging environment due to its versatile metabolism, efficiently utilizing nutrients that are less preferred by surrounding cells and adapting to anaerobic conditions through fermentation pathways and denitrification[19,20].

The metabolic plasticity of *P. aeruginosa* critically influences the antibiotic sensitivity[21]. For instance, aminoglycosides rely on the proton motive force generated during cellular respiration to enter bacterial cells[22], while fluoroquinolones depend on elevated oxidative phosphorylation and reactive oxygen species production for their bactericidal activity[23]. Conversely, other antibiotics such as polymyxins specifically target cells with low metabolic activity, as bacterial resistance mechanisms require active metabolic remodeling of the lipopolysaccharide layer in the cell envelope[24].

Respiratory tract infections caused by *P. aeruginosa* in patients with CF are often associated with the formation of biofilms[25]. Biofilms are characterized by structured communities of bacteria embedded in a self-produced extracellular

matrix, which provides protection from antibiotics and the host immune system. Moreover, they are characterized by a heterogeneous cell populations which includes both actively dividing as well as dormant cells, which can lead to differential drug effects depending on the mode of action of antibiotics[26]. For example, the polymyxin antibiotic colistin predominantly targets the metabolically dormant biofilm core, whereas fluoroquinolones or aminoglycosides primarily target the metabolically active peripheral biofilm population[27,28]. Recent *in vitro* studies demonstrated that nutrient supplementation enhanced aminoglycoside or fluoroquinolone efficacy by universally activating energy metabolism[21,29]. These findings illustrate the important role of considering nutrients and cellular metabolism. However, the clinical relevance of these potentiation strategies remains uncertain, as *P. aeruginosa* is known to reconfigure its metabolic processes even in the absence of environmental pressures[30].

The interplay between transient antibiotic resistance responses and the remarkable metabolic versatility of *P. aeruginosa* creates a particularly complex challenge for antibiotic treatment in the unique environment of the CF respiratory tract. This complexity is further amplified by the diverse microenvironments within CF lungs, which arises from differences in underlying CF pathology, rates of lung function decline, comorbidities, polymicrobial infections, and the compartmentalized structure of the lung[31]. The influence of adaptation to these varied conditions is reflected in the wide range of resistance mechanisms and metabolic specializations observed in clinical *P. aeruginosa* isolates[32–34]. Gaining a deeper understanding of how *P. aeruginosa* adapts to these distinct CF lung environments impacts antibiotic treatment and is a crucial step toward addressing recurring treatment failures and preventing chronic infections.

Studying bacterial growth and pharmacodynamic responses *in vitro*

In current clinical practice, antibiotic treatment selection is guided by standardized antimicrobial susceptibility testing through determination of the minimum inhibitory concentration (MIC). The MIC aims to quantify the lowest concentration of an antibiotic that inhibits visible bacterial growth under standardized conditions. Limitations of the MIC include the static nature of the

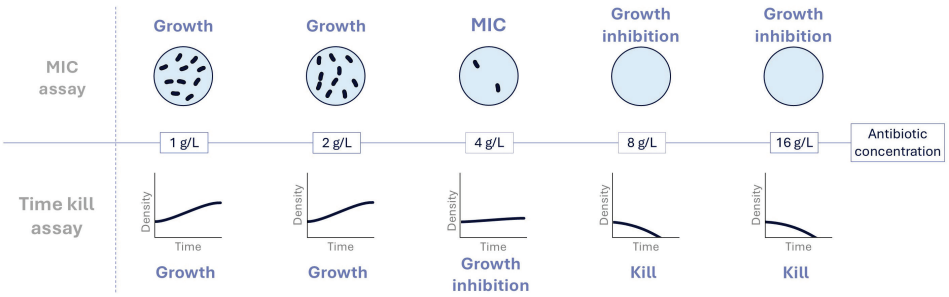

Figure 1. Difference between read-outs to evaluate the effect of antibiotics on bacterial growth and/or kill using minimal inhibitory concentration (MIC) and time-kill assays. MIC assays evaluate antibiotic drug effects at a fixed time point and can only assess growth inhibition. Time kill assays can characterize the dynamic effect of antimicrobial and can distinguish between growth inhibition and bacterial kill effects.

assay, i.e., after approximately 20 hours of incubation, the inability to characterize the pharmacodynamic response because bacterial kill cannot be measured, and the use of standard culture media which do not reflect the CF lung environment[12].

To overcome the limitations posed by the static endpoint measurements of MIC assays, time-kill assays can be employed. Time-kill assays monitor bacterial density over time and capture dynamic patterns of growth and killing[35]. Unlike MIC testing, these time-kill assays can reveal subtle shifts in bacterial population growth dynamics, offering valuable insights into transient responses to antibiotics (**Figure 1**), and regrowth patterns due to the development of antibiotic resistance. Typically in time kill assays, bacterial densities are quantified by counting the number of colony forming units (CFU), although surrogate readouts, such as constitutive bioluminescent bacteria[36] as used in this PhD thesis, can also be employed. Subsequently, mathematical pharmacodynamic modeling can be applied to these dynamic time kill profiles to infer concentration-effect relationships and uncover drivers of pharmacodynamic responses across different antibiotics and growth environments[37].

To better incorporate physiologically relevant conditions during *in vitro* assays, a synthetic CF sputum medium (SCFM) has been proposed in 2007 to mimic nutrient composition of CF sputum[38]. Culturing *P. aeruginosa* in SCFM more accurately reflects the physiological phenotypes of *P. aeruginosa* compared to standardized laboratory media[39,40], which consequently impacts *P. aeruginosa* responses to antibiotics[41–43]. Despite the improved physiological relevance of SCFM, performing MIC tests with a single medium composition falls short of capturing the extensive nutrient heterogeneity encountered in the CF lung. Additionally, nutrient-driven phenotypic factors influencing the antibiotic response of *P. aeruginosa* can be more accurately characterized using pharmacodynamic modeling (**Figure 2**). These changes in antibiotic response are often broadly categorized. For example, continued growth during antibiotic exposure is typically labeled as mutation-induced antibiotic resistance, while altered killing dynamics are frequently attributed to transient mechanisms[12]. However, the link between nutrient environment, bacterial phenotype and antibiotic effects points toward an intersection of underlying biochemical pathways that shape pharmacodynamic outcomes.

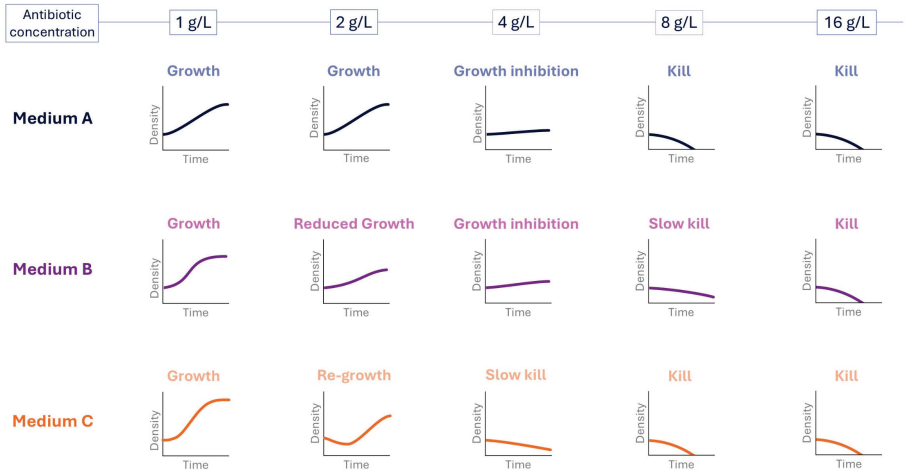

Figure 2. Examples illustrating the effect of media/nutrient conditions on phenotypic responses to antibiotic exposure. Although the dynamic responses differ, these conditions would yield similar outcomes in minimal inhibitory concentration (MIC) assays.

Studying the underlying genetic and biochemical pathways associated with altered antimicrobial drug effects is essential to further improve the translation of experimental pharmacodynamic findings into successful clinical treatment outcomes. This includes both the use of genetic characterization to identify driver mutations as well as the use of transcriptomic, proteomic and metabolomic techniques to identify downstream biochemical changes, in particular in relation to transient mechanisms that promote antibiotic tolerance in relation to the nutrient environment. Integrating data from these omics approaches can bridge this gap by providing a systems-level understanding of how environmental conditions and resulting phenotypic adaptations influence antibiotic effectiveness and the underlying molecular mechanisms[44], thereby facilitating the selection or optimization of experimental models that better reflect the clinical context. Accounting for the phenotypic context of antibiotic sensitivity testing will not only be critical for guiding treatment strategies, but also for improving preclinical testing of new antimicrobials[45]. To translate these insights into practical advances in *P. aeruginosa* respiratory tract infections, a structured and incremental approach is needed, beginning with the systematic evaluation of individual environmental components. This is the groundwork to elucidate the dynamic interplay between the CF lung microenvironment, *P. aeruginosa* metabolism, and antibiotic effects.

1.2. Thesis outline and scope

The impact of the phenotypic plasticity of *P. aeruginosa* and the distinct CF lung microenvironments on antibiotic drug effects and resistance remains poorly understood. To improve antibiotic treatment strategies for chronic respiratory infections caused by *P. aeruginosa* in CF patients, it is essential to gain deeper insights into how the infectious microenvironment shapes bacterial metabolism and antibiotic responses. In this thesis, we hypothesize that nutrient conditions play a central role as pharmacodynamic drivers and key determinants in the evolution of antibiotic resistance, ultimately affecting therapeutic outcomes. To summarize our current understanding regarding this hypothesis, **Chapter 2** (Section I) provides a comprehensive overview of the metabolic adaptations of *P. aeruginosa* to the diverse nutrient niches within the CF lung. These metabolic adaptations are further discussed in relation to their influence on antibiotic efficacy and their potential to explain the variability in treatment outcomes observed among patients. Building on this foundation, the thesis is structured into two key sections: examining nutrient-driven changes in antibiotic sensitivity (Section II), and investigating the influence of nutrients on antibiotic resistance evolution (Section III).

Section II: Nutrients shape antibiotic sensitivity

Section II investigates the role of physicochemical components of physiologically relevant habitats in the CF respiratory tract on antibiotic sensitivity. In **Chapter 3**, a study is described that systematically examines how specific *in vitro* nutrient conditions modulate the antibiotic pharmacodynamics of *P. aeruginosa*. To this end, single nutrients relevant to both *P. aeruginosa* metabolism and CF sputum are altered, and the resulting changes in pharmacodynamics are assessed across multiple antibiotic classes. **Chapter 4** explores how long-term adaptation of *P. aeruginosa* to anoxic conditions impacts antibiotic effects. An anoxic-adapted strain is generated through experimental evolution and its antibiotic response is compared to that of the parental strain.

Section III: Nutrients shape antibiotic resistance evolution

Section III aims to fill the current knowledge gap regarding how nutrient conditions are involved in the biochemical adaptation during development of

antibiotic resistance. In **Chapter 5**, the contribution of bacterial metabolism to antimicrobial resistance mechanisms is examined, along with how state-of-the-art metabolomics approaches can be used to map metabolic pathways that may be targeted to counteract resistance. **Chapter 6** investigates how nutrient conditions influence the evolution of antibiotic resistance in *P. aeruginosa*. Using adaptive laboratory evolution in single-nutrient media, specific nutrient-antibiotic combinations are examined to understand how they shape phenotypic resistance, fitness trade-offs, and mutational profiles across multiple antibiotic classes.

Section IV: General discussion and summary

In **Chapter 7**, the main findings of this thesis are discussed and summarized. The implications of these findings are considered, and next steps are outlined, particularly regarding characterizing nutrient niches in infection sites to better understand antibiotic responses and resistance emergence.

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How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 2

Nutrient-driven metabolic modulation of antibiotic efficacy in *Pseudomonas aeruginosa*

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In submission

Abstract

Pseudomonas aeruginosa is a versatile pathogen that can adapt its metabolism to diverse nutritional environments. It is a frequent cause of chronic respiratory infections, particularly in people with cystic fibrosis (pwCF). In pwCF, the infectious microenvironment is characterized by a unique and patient-specific nutrient environment. The nutrient-rich yet hypoxic mucus suppresses aerobic metabolism and promotes alternative metabolic pathways such as denitrification and fermentation, as well as the establishment of a biofilm-associated lifestyle. These adaptations promote sustained bacterial survival in the CF respiratory tract and may impair the efficacy of antibiotic therapy. This review summarizes how physiologically relevant nutrient environments drive metabolic changes in *P. aeruginosa* and subsequently its responses to antibiotics. We also discuss how CF-related pathophysiology may contribute to nutrient heterogeneity, potentially altering antibiotic effects. In conclusion, the complex interplay between nutrient availability, bacterial metabolism, and antibiotic response may provide both explanations and opportunities for tailoring antibiotic therapies in patients with chronic *P. aeruginosa* infections.

2.1. Introduction

Cystic fibrosis (CF) is associated with the formation of a thick, dehydrated mucus layer, hindering both oxygen (O_2) diffusion and waste clearance (**Figure 1A**), which often results in the establishment of chronic respiratory tract infections. *Pseudomonas aeruginosa,* a versatile Gram-negative opportunistic pathogen, is among the most predominant causes of chronic bacterial respiratory tract infections in adult patients with CF (pwCF)[1,2,3].

Mucus in the respiratory tract of pwCF provides a complex environment with energy substrates that can be efficiently utilized by *P. aeruginosa*[4] (**Figure 1B**). Mucus composition varies substantially between individual patients[5,6] and is spatially distributed across the compartmentalized lung[7]. *P. aeruginosa* is capable of adapting to these varying local environments due to its versatile and well-regulated metabolism[8,9]. In pwCF, *P. aeruginosa* is typically present in a biofilm lifestyle, wherein the biofilm extracellular matrix serves as a protective shield against both host immunity and antibiotics[10]. Mature biofilm structures impose constraints on O_2 and nutrient penetration, leading to the segregation of aerobic and anaerobic metabolic subpopulations which can impact antibiotic treatment effects[11] (**Figure 1C-D**).

Understanding the intricate relationship between diverse nutrient microenvironments and antibiotic responses is key to improving antibiotic treatment of chronic *P. aeruginosa* respiratory tract infections in pwCF. The current review aims to provide an overview of: (i) *P. aeruginosa* metabolic adaptation within clinically relevant CF lung microenvironments; (ii) the influence of changing nutrient environments on biofilm formation and antibiotic sensitivity; and (iii) the role of patient heterogeneity in nutrient diversity and treatment response.

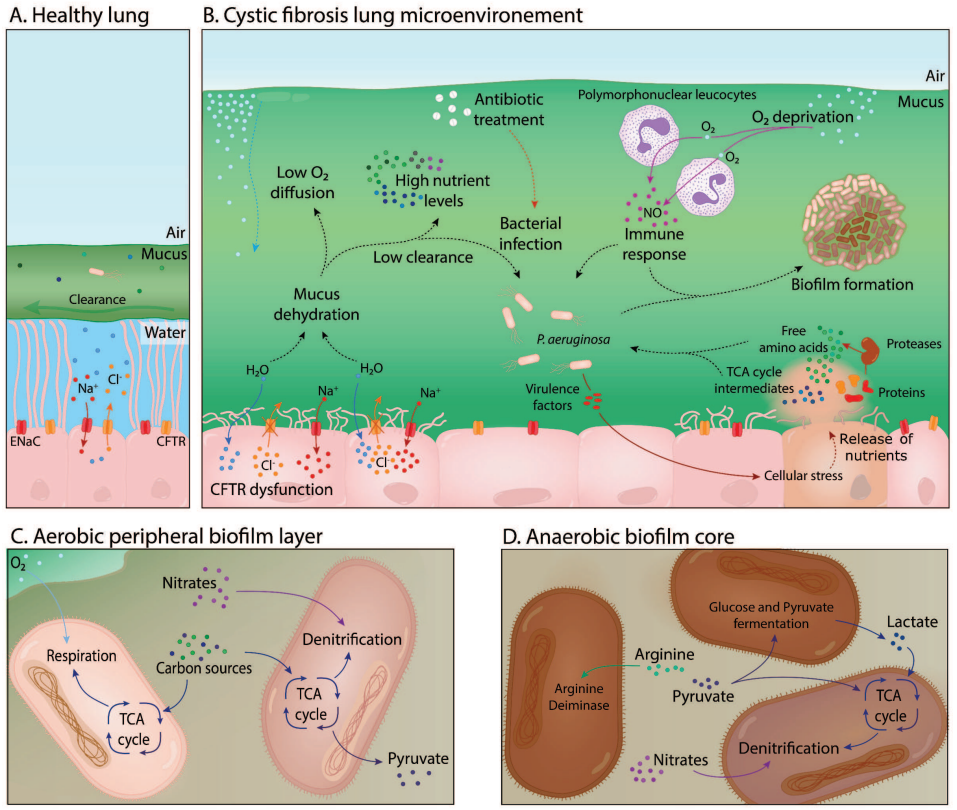

Figure 1. Overview of *P. aeruginosa* in the cystic fibrosis (CF) lung microenvironment. (**A**) demonstrates the efficient clearance of the mucus in healthy lungs with functional transmembrane proteins. (**B**) Accumulation of viscous dehydrated mucus and the microenvironmental influences in biofilm formation. Metabolic processes of cells in the (**C**) peripheral layer and (**D**) core of the biofilm.

2.2. Metabolic adaptation within the nutritional microenvironment in cystic fibrosis

The CF lung microenvironments are characterized by a large diversity in nutrients and O_2 levels. The following section describes *P. aeruginosa* metabolic pathways that are utilized or affected under these nutritional conditions.

Aerobic metabolism

Tricarboxylic acid (TCA) cycle

Amino acids and lactate are systematically increased in the CF lung and represent important nutrients for *P. aeruginosa* energy production through the TCA cycle[12,13,14]. These nutrients have different entry points into the TCA cycle, facilitating metabolic flux versatility (**Figure 2**). For instance, lactate is converted to pyruvate by lactate dehydrogenases (LldDE and LldA) to fuel the TCA cycle[15], while L-glutamate enters halfway in the cycle by glutamate dehydrogenases (GdhA and GdhB)[16]. The resulting electron carriers from the TCA cycle play a crucial role in oxidative phosphorylation (OXPHOS), supporting energy-demanding processes like extracellular matrix production during biofilm maturation[17,18]. Matrix-producing biofilm cells exhibit comparable TCA cycle activity to planktonic cells[19], underlining the high metabolic activity in the peripheral biofilm sub-population where nutrients and O_2 are still available. Finally, *P. aeruginosa* can also operate anaplerotic pathways in the TCA cycle, such as the pyruvate and glyoxylate shunt, if nutrients or O_2 become scarce. Shunting the TCA cycle reduces electron carrier production to maintain the redox balance during low energy demanding circumstances, such as the dormant biofilm core[19,20].

Glucose catabolism

Glucose levels are elevated in the CF respiratory fluid due to active stimulation of glucose leakage from lung epithelial cells and the induction of hemoptysis by *P. aeruginosa*[21–23]. Unlike many organisms, *P. aeruginosa* typically does not prefer glucose as primary carbon source in CF sputum due to the absence of glycolytic enzymes[5]. However, glucose catabolism remains crucial for the bacterial survival and pathogenicity, primarily through efficient production of pyruvate through the Entner-Doudoroff (ED) pathway[19,24]. *P. aeruginosa* employs a combination of enzymes from both the ED and Embden-Meyerhof-Parnas (EMP) pathway for a full carbohydrate degradation loop. The ED-EMP cycle is primarily used for anabolic functions, but also yields precursors for biofilm matrix and cell envelope production[19]. *P. aeruginosa* also actively secretes lipases and elastases to cleave macromolecules into metabolites suitable for the ED-EMP cycle[8,25]. For example, the peptidoglycan component N-acetylglucosamine present in CF sputum is processed within the ED-EMP system to be utilized intracellularly as a

carbon source[26,4]. The heightened levels of glycolytic substrates in CF sputum may contribute to the biofilm-aggregate structure observed in the CF lung. *P. aeruginosa* biofilms grown solely on glucose demonstrate reduced motility aggregate populations[27], suggesting an intricate interplay of glycolytic metabolism in shaping microbial community characteristics in the biofilm.

Amino acids and D-isoforms

Amino acids play a pivotal role in *P. aeruginosa* metabolism within the CF lung environment, serving as carbon or nitrogen sources and building blocks for proteins. Both *P. aeruginosa* and host immune cells contribute to the elevated amino acid concentrations in CF sputum through the excretion of peptidases[28,29]. The abundance of amino acids in CF sputum provides a favorable growth environment, whereby long-term evolution of pathogens in the CF lung can lead

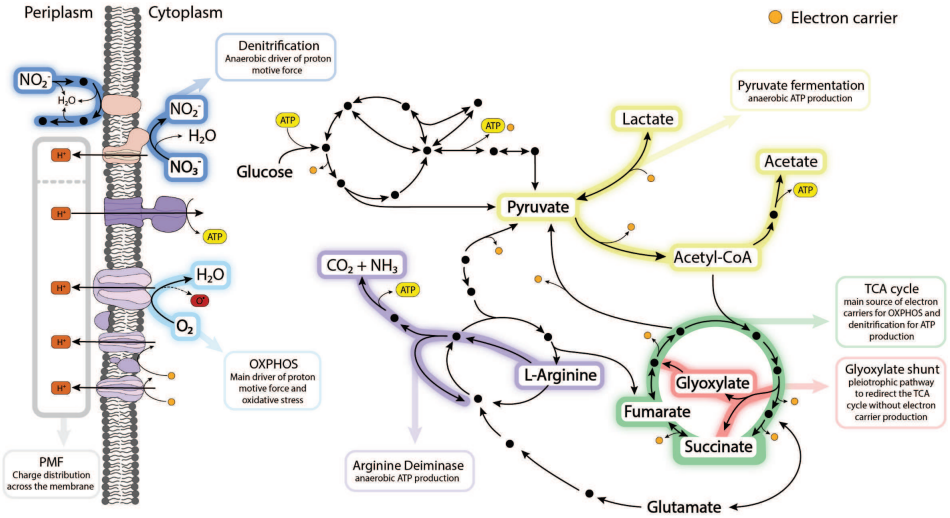

Figure 2. Central carbon metabolism of *P. aeruginosa* and the relation with antibiotic treatment. Metabolic map covering the EDEMP, TCA and urea cycle for electron carrier (orange) production for ATP (yellow) synthesis by oxidative phosphorylation (OXPHOS) and denitrification. Fermentation pathways are illustrated with colored arrows, glucose fermentation in blue, pyruvate fermentation in yellow and arginine fermentation in purple.

P. aeruginosa biofilm formation, with branched chain amino acids leucine, isoleucine and valine influencing *P. aeruginosa* growth rate and swarming motility[31,32]. Biofilm stimulation is isoform-dependent, as the D-isoform of leucine inhibits biofilm formation[32–36]. In contrast, other studies reported D-amino acid supplementation did not significantly improve survival outcomes in mice[35], and anti-biofilm effects of D-isoforms disappeared after several days of incubation[37]. These conflicting results highlight the relevance for further studies into the driving mechanisms of amino acids in *P. aeruginosa* infection in the CF lung.

Anaerobic metabolism

The dehydrated and thick mucus layer in the CF lung reduces O_2 diffusion. Levels of O_2 are further reduced by neighboring cells, such as lung epithelial cells and polynuclear monocytes (PMNs)[38,39]. This O_2 restriction contributes to the establishment of fully anaerobic microenvironments within the mature biofilm structure. In response to these O_2 constraints, *P. aeruginosa* employs adaptive strategies, utilizing two fermentation pathways and shifting from O_2 to nitrates as electron acceptors.

Glucose and pyruvate fermentation

The fermentation of glucose comprises two steps: the initial conversion of glucose to pyruvate, followed by the subsequent fermentation of pyruvate into lactate, acetate and succinate[40]. Glucose fermentation to pyruvate is typically influenced by redox constraints[41]. To overcome redox imbalances during anaerobic fermentation, *P. aeruginosa* actively produces radical-scavenging phenazines[41,42,43]. The NADH-dependent conversion of lactate to pyruvate conversion also preserves the cellular redox balance by limiting electron accumulation[41,42]. The emphasis on pyruvate metabolism in anaerobic conditions becomes evident by the increase in total biofilm biomass upon pyruvate supplementation and biofilm dispersion after pyruvate deficiency[44,45]. Efficient cross-feeding of pyruvate and lactate over the O_2 gradient demonstrates the cooperative metabolic activity between the biofilm sub-populations[15,46], and the role of carbon sources for maintaining matured biofilm structures.

Arginine deiminase

Arginine, an amino acid favored by *P. aeruginosa* as a carbon source, plays a crucial role in anaerobic metabolism and biofilm development. Arginine serves

as an energy source, undergoing fermentation through deiminase enzymes[32]. The transcription of the *arcCBAB* operon encoding for arginine deiminase enzymes is upregulated during biofilm formation, indicating its importance in this process[17]. The utilization of arginine deiminase enzymes is a relatively inefficient pathway for energy production, reducing motility and promoting the transition towards a static biofilm phenotype[47,48,32]. While fermentation maintains energy levels for cellular maintenance in anaerobic environments, it does not provide the efficiency to enable net growth[40,47], which might explain the presence of a dormant sub-population within the biofilm core. The role of arginine extends beyond serving as an energy source in anaerobic conditions. It also acts as a precursor of gene-modulating polyamines that contribute to the formation of the extracellular matrix of biofilms[49]. The central role of arginine in biofilm maturation is further supported by sensory domains, inducing *Pseudomonas Putida* biofilm formation in the presence of exogenous arginine[50].

Denitrification

P. aeruginosa capitalizes on denitrification for proliferation in anaerobic conditions. This process substitutes O_2 with nitrate (NO_3-) and nitrite (NO_2-) as electron acceptors (Figure 1D)[51,52]. This shift facilitated by the ample availability of these compounds in CF mucus, which also enables activation of the denitrification pathway despite the presence of O_2. The high abundance of these nitrates does not limit the utilization of the denitrification pathway within anaerobic environments[5,53]. *P. aeruginosa* also employs denitrification enzymes to neutralize nitric oxide (NO) produced by immune[14,54] and to mitigate ROS production by distributing the electron flow across the respiratory and denitrification pathways in O_2-rich conditions[8,40,55]. The functionality of denitrification is iron-dependent[51]. Reduced transcription of denitrification proteins hampers anaerobic metabolism under iron scarcity[56], whereas sufficient levels of iron stimulate anerobic metabolism and biofilm development[57–60]. This underscores the critical role of denitrification in the maturation of biofilm and adaptation of anaerobic sub-populations in the core, highlighting how metabolic processes are intricately linked to multiple nutrients in the surrounding microenvironment. This metabolic coordination is also regulated at the transcriptional level. The *Anr* transcriptional regulator, which controls denitrification enzymes, also has conserved regulatory effects in central carbon metabolic pathways[40,55]. For instance, under anaerobic yet nitrate-rich

conditions, arginine is no longer a preferred carbon source[5,47]. This metabolic heterogeneity is reflected in biofilm structures, where different metabolic pathways dominate at varying biofilm depths depending on nutrient availability[15,61]. Consequently, the metabolic adaptation of *P. aeruginosa* at the infection site is influenced by the overall nutritional composition, including O_2, metals, salts, and carbon sources. This highlights both the flexibility and diversity of *P. aeruginosa* metabolism within the CF lung, underscoring the importance of understanding the complex interplay between bacterial metabolism and the nutritional environment for effective therapeutic interventions.

2.3. Nutrient-driven effects of metabolic adaptation on antibiotic sensitivity

In this section, we discuss the role of nutrients in the metabolic activity of *P. aeruginosa*, and how this influences antibiotic sensitivity. Nutrient-limited environments typically induce low metabolic activity, which is generally linked to reduced activity of antimicrobials, since antibiotics often target energy-demanding cellular processes during cell division[62]. This includes processes such as DNA replication (e.g., fluoroquinolones), protein synthesis (e.g., aminoglycosides) and cell wall synthesis (e.g., β-lactams). In contrast, polymyxins are more effective in eradicating metabolically inactive cells[63]. An overview of specific nutrients present in the CF lung and their modulatory role on antibiotic efficacy is summarized in **Table 1**.

Fluoroquinolones

Fluoroquinolones require oxidative stress for effective bacterial killing in addition to their primary mode of action through inhibition of DNA gyrase and topoisomerase IV[82–84]. Oxidative stress primarily stems from aerobic metabolic activity, i.e., TCA cycle and OXPHOS, which spearhead ROS production. These processes can be suppressed in low oxygen environments, for example, in the O_2-and nutrient-deprived core of a biofilm, in addition to phenazine-mediated redox balancing mechanisms in anaerobic environments to reduce oxidative damage[61]. To illustrate, in *P. aeruginosa* biofilms, enhanced ciprofloxacin tolerance has been

Table 1. Nutrient supplements which change antibiotic sensitivity through metabolic changes

Antibiotic	Antimicrobial effect	Nutrient environment	Metabolic mechanism	Ref.
Ciprofloxacin	Potentiation	O_2	Electron transport chain	64
		Malic acid	TCA cycle activity	65,66
		Arginine	n.r.	67,68
	Reduction	n.r.	Denitrification	69–71
		Starvation	Dormancy	72
Tobramycin	Potentiation	O_2	n.r.	73
		Fumarate	Proton motive force, TCA cycle	74,75
		Glutamate and succinate	Proton motive force, TCA cycle	76
		bicarbonate	Alkaline pH	77
		Arginine	Alkaline pH	67,78,79
	Reduction	Glyoxylate	Proton motive force, TCA cycle	74
		n.r.	Denitrification	53
Meropenem	Reduction	Starvation	Oxygen radicals	80
Colistin	Potentiation	Nitrate	Anaerobic metabolism	63
	Reduction	Glucose	"Osmotic homeostasis"	81
		Formate	n.r.	81

n.r. = not reported

To illustrate, in *P. aeruginosa* biofilms, enhanced ciprofloxacin tolerance has been observed in metabolically inactive cells as compared to metabolically active cells[85]. Consequently, the supplementation of O_2, i.e., to promote aerobic respiration, improves ciprofloxacin efficacy against *in vitro* grown biofilms[64]. Similar ciprofloxacin potentiation was observed when supplementing with organic acids to increase TCA cycle activity[65,66]. By using metabolic shunts as a safeguard against oxidative stress while preserving anabolic flexibility[20], *P. aeruginosa* demonstrates a form of metabolic defense against fluoroquinolone action. This adaptability becomes particularly evident in the nitrogen rich CF lung environment, where *P. aeruginosa* shifts from OXPHOS to denitrification, thereby reducing oxidative stress and increase tolerance to fluoroquinolones while maintaining metabolic activity[69–71]. The reduced fluoroquinolone

susceptibility by the activation of anaerobic metabolism is not observed when anaerobic nutrients such as NO_3 and arginine are supplemented. In fact, the addition of arginine and NO_3 demonstrated enhanced ciprofloxacin activity in mature biofilm cultures, while no enhancement was observed in young or alginate-grown biofilms[67,68].

Aminoglycosides

Aminoglycosides penetrate bacterial cells through membrane pores and block protein synthesis by attaching to ribosomal proteins. Their entry relies on the electric potential across the cell membrane, driven by the proton motive force (PMF) during OXPHOS[86,87]. Stimulating the PMF by elevating OXPHOS activity through O_2 supplementation can enhance the effectiveness of tobramycin[73]. Similarly, the supplementation of fumarate increases the electron transport through the elevated TCA cycle, improving aminoglycoside action[74,75]. However, supplementing with glyoxylate activates the glyoxylate shunt, which reroutes the TCA cycle and shifts the balance between OXPHOS and denitrification. This reduces the PMF and consequently decreases susceptibility to tobramycin[20,8,53,74]. This metabolic adaptation is also observed in biofilms exposed to tobramycin, where cells in aerobic biofilm regions shift to denitrification upon exposure to tobramycin[53].

The transmembrane pH gradient is another component of the PMF that plays a crucial role in aminoglycoside activity. The acidic CF lung environment lowers both the net positive charge of tobramycin and the PMF of *P. aeruginosa*, thereby reducing aminoglycoside effectiveness[88]. This can be counteracted by increasing the pH through bicarbonate supplementation, which has shown to enhance tobramycin effect[77]. Adjusting the pH showed limited benefits for aminoglycoside treatment of biofilm, potentially due to the natural acidic pH-gradient in biofilm structures from accumulated extracellular DNA[77,78,88,89]. In contrast, arginine supplementation has shown promise in enhancing aminoglycoside efficiency in biofilms, due to metabolically induced pH increase[67,78,79]. Studies involving the use of 3D cultured lung cells have shown that a combination of altered pH, transmembrane potential, and carbon metabolism enhance aminoglycoside effect[76]. Alkalinizing the intracellular environment and increased TCA cycle activity through pyruvate metabolism increased the PMF-mediated aminoglycoside uptake. Furthermore, enriching the 3D culture media

with succinate and glutamate significantly improved aminoglycoside-mediated eradication of biofilms.

β-Lactam antibiotics

β-Lactam antibiotics exert their bactericidal effect by depleting cell wall building blocks through inhibition of cell envelope precursor synthesis. Primary mechanisms modulating β-lactam resistance typically have a genetic basis, including porin modifications, overexpression of efflux pumps, and inactivating β-lactamase enzymes[90]. Whereas the primary mechanisms of action and resistance to β-lactam antibiotics are generally stable across different environmental and metabolic conditions, secondary effects such as the induction of oxidative stress are closely linked to both[91–94]. The bactericidal activity of β-lactams can be potentiated by the interaction of ferrous ions with reactive oxygen species (ROS), which are generated as a result of the elevated metabolic activity associated with peptidoglycan recycling[95]. This oxidative mechanism aligns with observations that meropenem is more effective against *Pseudomonas aeruginosa* strains with compromised antioxidant defenses[80]. In contrast, activation of stress responses such as the stringent response can enhance antioxidant capacity prior to antibiotic exposure, thereby promoting antibiotic tolerance[80,96]. The stringent response also plays a key role during nutrient limitation in the biofilm core[97], contributing to biofilm physiology and potentially reducing susceptibility to β-lactams. Although the influence of the nutritional environment on the early bacterial response to β-lactams is not yet fully understood, it may be an important factor in shaping *P. aeruginosa* sensitivity.

Polymyxins

Polymyxins are polypeptide antibiotics that disrupt the bacterial cell envelope. Unlike many other antibiotics, polymyxins are particularly effective against dormant cell types that lack the high metabolic activity required for cell envelope remodeling[63]. These metabolic demands for lipopolysaccharide modifications are more readily supported in nutrient-rich environments, which can lead to a reduction in binding sites for colistin due to alterations of the lipid A component of lipopolysaccharide[85,98,99]. While such lipid A remodeling typically imposes a fitness cost in other Gram-negative bacteria, *P. aeruginosa* appears to tolerate these modifications without significant fitness penalties[99–101]. Nonetheless,

colistin susceptibility in *P. aeruginosa* can be influenced by the nutrient environment. For example, the carbon sources glucose and formate modulate antibiotic effects while not directly channeled into the central energy-generating pathways[81]. Glucose has been suggested to reduce susceptibility by alleviating osmotic stress, while formate induces a sensitizing effect through an as-yet undefined mechanism. These findings illustrate how *P. aeruginosa* leverages its metabolism to adapt to antibiotic exposure in ways that are uncoupled from core energy metabolism. However, such metabolic adaptations likely depend on nutrient-rich conditions that maintain energy homeostasis via alternative substrates.

2.4. Differences in the nutritional environments between and within patients

Differences in nutrients may impact the response of *P. aeruginosa* to antibiotics can occur at different biological scales, contributing to variability within and between patients. In the previous sections, we highlighted how nutritional diversity within the CF lung influences *P. aeruginosa* phenotype and antibiotic sensitivity at the cellular level, explaining heterogeneity at the cellular level (**Figure 3A**). Within the lung, accumulation of mucus and macronutrients can further contribute to this nutrient diversity. Nutrient conditions vary substantially across different lung regions due to varying host-pathogen interactions and oxygen availability[102] (**Figure 3B**). Patient-specific differences such as those related to disease severity, inflammation, comorbidities and microbial colonization significantly impact the nutrient microenvironments[104–109] (**Figure 3C**). In this section, we explore how nutrient heterogeneity at the tissue and patient level may further contribute to differences in antibiotic treatment response.

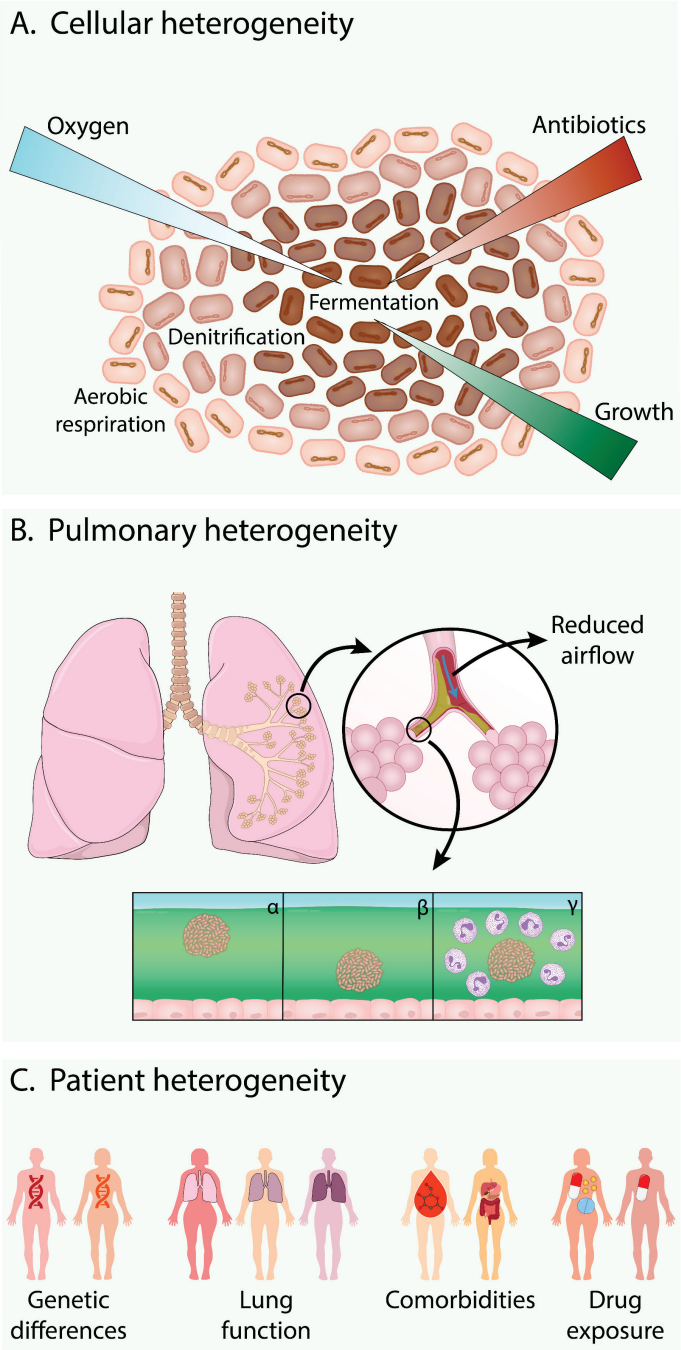

Figure 3. Multiscale heterogeneity contributing to variation in antibiotic treatment response in (**A**) patients, (**B**) site of infection, and (**C**) bacterial biofilm structure.

Pulmonary heterogeneity

The compartmentalization of the human lung creates a diverse microenvironment that significantly influences pathogen-host interactions[102]. One notable example is the respiratory zone, which is densely populated with PMNs and, as a result, experiences a pronounced depletion of O_2 from the typically aerobic zone[109]. It has been suggested that biofilm aggregates encased by PMNs no longer exhibit an oxygen gradient but instead experience uniform hypoxia[110]. Microbes originating from these hypoxic areas can adapt to become intolerant to O_2[111], a shift that will directly affect their metabolic processes and likely their response to antibiotics. For example, proposed strategies focusing on increasing aerobic respiration may have limited or even counterproductive effects on these strict anaerobic cells, especially when compared to *P. aeruginosa* lineages that have evolved increased aerobic respiration during long-term adaptation to the CF lung[25].

The O_2 depleted by PMNs is partly used for ROS production as a pathogen eradication mechanism, but also inducing oxidative stress in nearby host and microbial cells. This oxidative stress not only increases nutrient availability through cell lysis and epithelial cell nutrient leakage[112], but also primes *P. aeruginosa* by activating stress responses prior to antibiotic treatment. The pre-activation of these stress responses can undermine the secondary effects of antibiotics that depend on ROS production, such as fluoroquinolones and β-lactams, reducing antibiotic effectiveness[113].

These observations underscore the critical need to consider the role of the compartmentalized lung in nutrient availability, oxygen levels, and immune cell activity in *P. aeruginosa* treatment response. Understanding the intricate dynamics of the different CF lung microenvironments and microbial adaptation offers a pathway to more effective treatment approaches, potentially including the strategic manipulation of microbial metabolism to enhance antibiotic efficacy.

Patient heterogeneity

There are nearly 2000 possible mutations of the CF transmembrane regulator gene that cause CF, leading to a broad spectrum of disease severities[114]. These mutations result in diverse manifestations in CF pathophysiology which

eventually contribute to significant differences in lung function decline[115,116]. Progressive lung function decline caused by inflammation results in higher levels of free amino acids, nutrients that are preferred by *P. aeruginosa*[107]. The more severe lung damage in chronically infected pwCF is often accompanied by *P. aeruginosa* adaptations that further enhance its metabolic dominance in the inflamed environment[107,108,117]. These metabolic changes also include the shift from aerobic respiration toward denitrification[118], a transition that is associated with decreased antibiotic susceptibility. This suggests that changes in the nutrient environment and associated metabolic adaptations may be important considerations in treatment decision-making. Such factors may be particularly relevant in the treatment strategy during the substantial changes in lung function that occur at the transition from childhood to adulthood[119].

Diabetes mellitus is one example of an important and frequent comorbidity in pwCF[119], which further exacerbates the elevated glucose concentrations typically observed in the CF lung[120,121]. Elevated glucose levels in the CF lung have been repeatedly associated with an increased risk of developing respiratory infections[122,123,124]. In vitro studies have demonstrated that glucose induces metabolic shifts and increase biofilm formation in *P. aeruginosa*, which in turn reduces levofloxacin susceptibility[125]. Glucose also plays a key role in epithelial cells and PMNs, particularly in anaerobic conditions where it is fermented into lactate. The subsequent rise in lactate levels has been suggested as a biomarker for pulmonary exacerbations[13,23,126]. However, the lack of significant decrease in lactate levels following antibiotic treatment raises questions about its utility as a reliable biomarker[127]. Nonetheless, the sustained lactate levels indicate that *P. aeruginosa* continues to access lactate and glucose before, during, and after antibiotic treatment. This demonstrates that glucose serves as an important substrate for *P. aeruginosa*, despite not being one of its preferred carbon sources.

2.5. Considering nutrients in antimicrobial susceptibility testing

Current clinical decisions regarding antibiotic therapy are based on antimicrobial susceptibility testing, which typically uses standardized nutrient

and oxygen-rich conditions that do not reflect the *in vivo* nutrient environment shaped by CF-related pathophysiological factors. By integrating physiologically relevant growth conditions that closely mimic the CF-specific environments, the predictive accuracy of antibiotic effects may be improved[128]. Combining these nutrient-relevant conditions with recent advancements in the development 3D biofilm[25] and host-microbe[157] models promises a more accurate translation of laboratory findings into clinical outcomes.

2.6 Opportunities for antimicrobial drug development

The intricate relationship between metabolic activity and *P. aeruginosa* antibiotic sensitivity presents a promising avenue for developing a new class of antimicrobials that target bacterial metabolism[129]. These novel antimicrobials could aim to inhibit the specific metabolic pathways that pathogens exploit to evade or tolerate conventional antibiotics like aminoglycosides and fluoroquinolones. We have described how the metabolic shift of *P. aeruginosa* toward anaerobic energy-generating pathways reduces antibiotic uptake and decreases ROS production, which are essential to the activity of these antibiotics. By specifically targeting these anaerobic pathways in combination therapies, metabolism-targeting antimicrobials could effectively block bacterial escape routes from antibiotic treatments[130]. Unlike nutrient supplementation that relies on activation of aerobic metabolism, combination therapy with metabolism-targeting antimicrobials will be a consistent strategy to combat antibiotic resistance within the diverse oxygen gradients of the CF lung. The success of these therapeutics hinges on a deep understanding of pathogen metabolism within physiologically relevant microenvironments. Selecting metabolism-targeting candidates from drug libraries can only facilitate the development of successful candidates if the pathogens are studied in screening models that accurately represent the infection site[131,132]. The failure of many promising compounds during the development process often stems from a lack of consideration of physiological relevance of screening models[133], underscoring the importance of this strategy in the fight against antibiotic-resistant infections.

2.7. Conclusion

There is a complex interplay of nutrients in the CF lung environment, metabolic adaptations of *P. aeruginosa* and resulting consequences for antibiotic treatment efficacy. Various nutritional environments relevant to the CF lung influence antibiotic efficacy. These insights highlight the importance of further considering the CF lung microenvironment and its impact in order to refine susceptibility testing and treatment strategies, although characterization of the nutrient environment in patients remains challenging.

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Section II

Nutrients shape antibiotic sensitivity

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 3

Nutrient conditions affect antimicrobial pharmacodynamics in *Pseudomonas aeruginosa*

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Microbiology Spectrum, 2025, 13(1), e01409-24

Abstract

The infectious microenvironment in chronic respiratory tract infections is characterized by substantial variability in nutrient conditions, which may impact colonization and treatment response of pathogens. Metabolic adaptation of the CF-associated pathogen *Pseudomonas aeruginosa* has been shown to lead to changes in antibiotic sensitivity. The impact of specific nutrients on the response to antibiotics is, however, poorly characterized. Here, we investigated how different carbon sources impact the antimicrobial pharmacodynamic responses in *P. aeruginosa*. We evaluated the effect of six antibiotics (aztreonam, ceftazidime, ciprofloxacin, colistin, imipenem, tobramycin) on *P. aeruginosa* cultured in a basal medium enriched for seven different carbon sources (alanine, arginine, aspartate, glucose, glutamate, lactate, proline). Pharmacodynamic responses were characterized by measuring time-kill profiles for a bioluminescent *P. aeruginosa* PAO1 *Xen41* strain. We show that single-nutrient modifications minimally affected bacterial growth rate. For specific nutrient-antibiotic combinations, we find relevant alterations in antibiotic sensitivity (i.e., EC_{50}) and the maximum drug effect (E_{max}), in particular for ciprofloxacin, colistin, imipenem and tobramycin. The most pronounced effect was observed for tobramycin, where glucose was found to reduce the EC_{50} (0.5-fold) while lactate-enriched conditions led to a 4.3-fold increase in EC_{50}. Using pharmacokinetic-pharmacodynamic simulations, we illustrate that the magnitude of the nutrient-driven pharmacodynamic changes impact treatment for clinical dosing strategies of tobramycin. In summary, this study underscores the impact of nutrient composition on antimicrobial pharmacodynamics, which could potentially contribute to observed variability of antimicrobial treatment responses in CF patients.

Importance

Chronic respiratory tract infections in cystic fibrosis patients present significant challenges for antibiotic treatment due to the complexity of the respiratory environment. This study investigated how variations in nutrient levels, altered during chronic infections, affect pathogen response to antibiotics in an experimental setting. By simulating different nutrient conditions, we aimed to

uncover interactions between nutrient availability and antibiotic sensitivity. Our findings provide critical insights that could lead to more effective treatment strategies for managing chronic respiratory tract infections in cystic fibrosis patients, while also guiding future research in improving treatment methodologies.

3.1. Introduction

Cystic fibrosis (CF) associated lung infections are facilitated by a complex infectious microenvironment involving a dense mucus layer harboring a diverse array of potential microbial nutrients[1]. Antibiotic treatment in patients with CF often yields unpredictable outcomes and aligns poorly with routine antimicrobial susceptibility testing[2,3]. Profound variability in microbial nutrients is observed within the chronic infectious environment, both across and within patients[4,5]. Unlike many other bacterial pathogens, *Pseudomonas aeruginosa* prioritizes the utilization of a wide array of carbon sources over glucose, including alanine, arginine, aspartate, glutamate, proline, and lactate[6,7]. This metabolic versatility may explain its pervasive presence in chronic CF-associated infections, and provides a competitive advantage during antibiotic treatment[8–10].

Alterations in metabolic processes associated with differences in available nutrients may impact response to antibiotic treatment in *P. aeruginosa*[11–13]. For example, nutrient deprivation prevents cell wall modifications due to its high energy demand, enhancing the effect of cell wall targeting antibiotics (e.g., polymyxins and β-lactams)[14–16]. The supplementation of metabolites to activate energy production through aerobic respiration in nutrient-deprived environments can increase sensitivity towards fluoroquinolones and aminoglycosides[17–19]. While these changes illustrate the modulatory role of deprived nutrient conditions and microbial metabolism on the response to antibiotics, insights into the contribution of nutrients relevant to CF lung microenvironments remain limited.

To assess the effects of nutrient conditions on antimicrobial pharmacodynamics (PD), conventional readouts such as minimum inhibitory concentrations (MIC) have important limitations, as this is a static composite measure. More comprehensive characterization of changes in the pharmacodynamic response

to antibiotics can be achieved through time kill studies, which monitor bacterial densities over time when exposed to antibiotics, allowing the evaluation of bacterial growth, antibiotic-associated killing, and adaptation effects[20,21]. Although time-kill studies provide these valuable insights, they remain limited in their throughput and the number of time points at which data can be collected[22]. The use of bacterial strains carrying luminescent reporters allows real-time monitoring of bacterial growth and killing dynamics during antibiotic exposure[23,24]. The resulting profiles can be analyzed using mathematical pharmacodynamic models to obtain further quantitative insights into PD relationships. As such, the use of luminescence-based time kill studies in combination with quantitative pharmacodynamic models is well-suited for comprehensively assessing the effects of nutrient conditions on antibiotic response.

In the current study, we aimed to systematically evaluate the impact of a wide range of CF sputum-relevant carbon sources on antimicrobial time-kill responses in *P. aeruginosa*. The nutrients evaluated included alanine, arginine, aspartate, glutamate, lactate, proline, and glucose. These nutrient-associated effects were evaluated for six antibiotics commonly used for respiratory tract infections in CF, including aztreonam, ceftazidime, ciprofloxacin, colistin, imipenem, and tobramycin. We assessed the bacterial growth/kill time course profiles using extensive time-kill studies with a modified *P. aeruginosa* PAO1 strain carrying a constitutively active luminescent reporter. This strain was subsequently used to infer PD parameters and perform pharmacokinetic-pharmacodynamic (PK-PD) simulations to demonstrate the potential clinical impact of nutrients on antimicrobial PD.

3.2. Materials and Methods

Culture media and bacterial strain

A basal medium was prepared consisting of physiologically relevant concentrations of amino acids in synthetic CF sputum as described previously[7], calcium and magnesium adjusted 0.11 M phosphate buffer, ammonium chloride, potassium nitrate, ferrous sulfate, Basal Medium Eagle 1x vitamins, and trace metals. The pH of the basal medium was confirmed to be 7.4, and was verified after addition of nutrients and filter sterilization. The specific concentrations of

all medium components are listed in **Table S1**. We then prepared 7 unique nutrient-specific media for each of the carbon sources used in this study, including alanine, arginine, aspartate, glutamate, glucose, proline, and lactate. Each of these nutrients was added separately to the basal medium in excess at a concentration of 15 mM. The *P. aeruginosa* bioluminescent strain PAO1 Xen41 (Revvity Inc., Waltham, MA, USA) was used in all experiments. The promoterless insertion of the *luxCDABE* cassette into the chromosomal genome resulted in a linear relationship between luminescence in relative light units (RLU) and CFU/mL (**Figure S1**)[23,24].

Antibiotics

Antibiotic stock solutions were freshly prepared on the day of the experiment and diluted to desired concentrations using an Opentrons OT-2 (Opentrons Inc., New York, NY, USA) liquid handling system. Aztreonam and ceftazidime pentahydrate were purchased from Thermo Fisher Scientific (Breda, The Netherlands). Ciprofloxacin, imipenem monohydrate, and tobramycin were purchased from Chem-Impex International (Wood Dale, IL, USA). Colistin sulfate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Experimental workflow

Time-kill assays were conducted by culturing *P. aeruginosa* in each of the nutrient-specific media formulations and exposing the cultures to 6 different antibiotics. We tested 9 different serially diluted concentrations in a microtiter plate format, centered around their minimal inhibitory concentrations (**Figure 1**). All experiments were conducted at 37 °C and with shaking at 150 rpm.

The PAO1 Xen41 strain was streaked on LB agar plates and incubated overnight. One colony was transferred to a nutrient specific media formulation (4 mL) and cultured overnight. The liquid cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 before inoculation, corresponding to an approximate bacterial concentration of $5*10^6$ CFU/mL. The bacterial inoculum (50 µL) was added to fresh medium with antibiotics (150 µL) in a white 96-well microtiter plate.

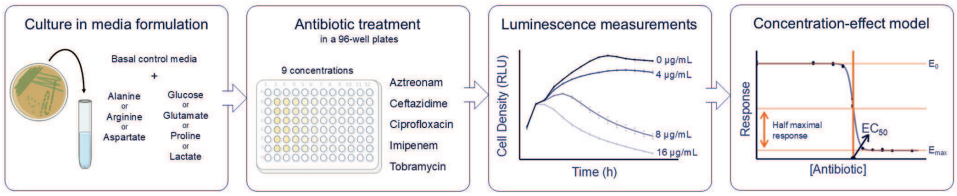

Figure 1. Experimental approach. The experiment started with by a liquid culture in the media formulation containing 1 or none of the nutrients of interest. The population was diluted to the starting density and treated with 9 concentrations of antibiotic while the luminescence was determined every hour in relative light units (RLU). A four parameter log-logistic function was fitted on the area under the curve or growth rate per antibiotic concentration to determine the upper limit (E_0), lower limit (E_{max}), and half-maximal effective concentration (EC_{50}).

After inoculation, microtiter plates were transferred to a Liconic StoreX STX44 incubator (Mauren, Principality of Liechtenstein) for incubation (95% relative humidity). A Peak Analysis and Automation KX-2 Laboratory Robot (Hampshire, United Kingdom) transferred the microtiter plate every hour between the incubator and the BMG Labtech Fluostar Omega microplate reader (Ortenberg, Germany) for time-course data acquisition. The density of viable bacteria was determined by measuring luminescence, quantified as relative light units (RLU).

Data processing and analysis

All data preprocessing and analyses are performed using R. To evaluate fitness differences between growth media, the maximal population growth rates (μ_{max}) and the maximal population density (N_{max}) under antibiotic free culture conditions were calculated using the all splines function from the grofit package[25]. Differences in growth parameters in the studied media formulations compared to the basal media were assessed by the Dunnett's Test from the DescTools R package[26].

To quantify drug effects, the total bacterial burden was determined by calculating the area under the curve (AUC) of the RLU between 1 and 15 hours of incubation (**Figure S2**). The resulting AUC values were then used to quantify

pharmacodynamic parameters. We fitted for each antibiotic-nutrient combination the mean (n=3) AUC to the antibiotic concentration ([AB]) using a four parameter log-logistic (LL.4) function from the drc R package (**Equation 1**)[27]. This function includes parameters for the hill coefficient (n_H), the lower limit (E_{max}), the upper limit (E_0), and the relative half-maximal effective concentration (EC_{50}). The difference in relative EC_{50} among culture conditions was quantified using the 95% confidence interval.

$$AUC([AB]) = E_{max} + \frac{E_0 - E_{max}}{1 + e^{n_H(\log([AB]) - \log(EC_{50}))}} \qquad (1)$$

Pharmacokinetic-pharmacodynamic (PK-PD) simulations

We used a previously published pharmacokinetic (PK) model for tobramycin to perform PK-PD simulations[28]. We simulated the clinical concentration-time profiles for a typical dose of 3.3 mg/kg of intravenous tobramycin, administered every 8 hours (**Table S2**). Interpatient variability for the parameters was derived from published interquartile ranges. Antibiotic PD was described by first estimating growth/kill rates for each antibiotic concentration, which were subsequently fitted to a pharmacodynamic sigmoidal function relation antibiotic growth/kill rate to antibiotic concentration. The growth rates where determined by determining the slope of the phase of the luminescence time kill curve where the drug effect occurred (**Figure S6**), using the grofit package.

3.3. Results

Nutrient-dependent shift in antibiotic sensitivity.

We cultured *P. aeruginosa* under various nutrient conditions in the presence of different antibiotics to investigate the effect of nutrients on the pharmacodynamic (PD) response. To summarize the bacterial response kinetics–encompassing growth enhancement, suppression, or killing during antibiotic treatment–we calculated the AUC of the luminescence time course profiles. We then regressed the AUC values against antibiotic concentrations using a sigmoidal Emax model, allowing us to visualize differences in the pharmacodynamic response across conditions (**Figure 2**). Overall, these analyses revealed significant effects of nutrients on the antibiotic concentration required to achieve

50% of the total antimicrobial effect (relative EC_{50}), and the steepness of the concentration-response profiles (**Figure S3**).

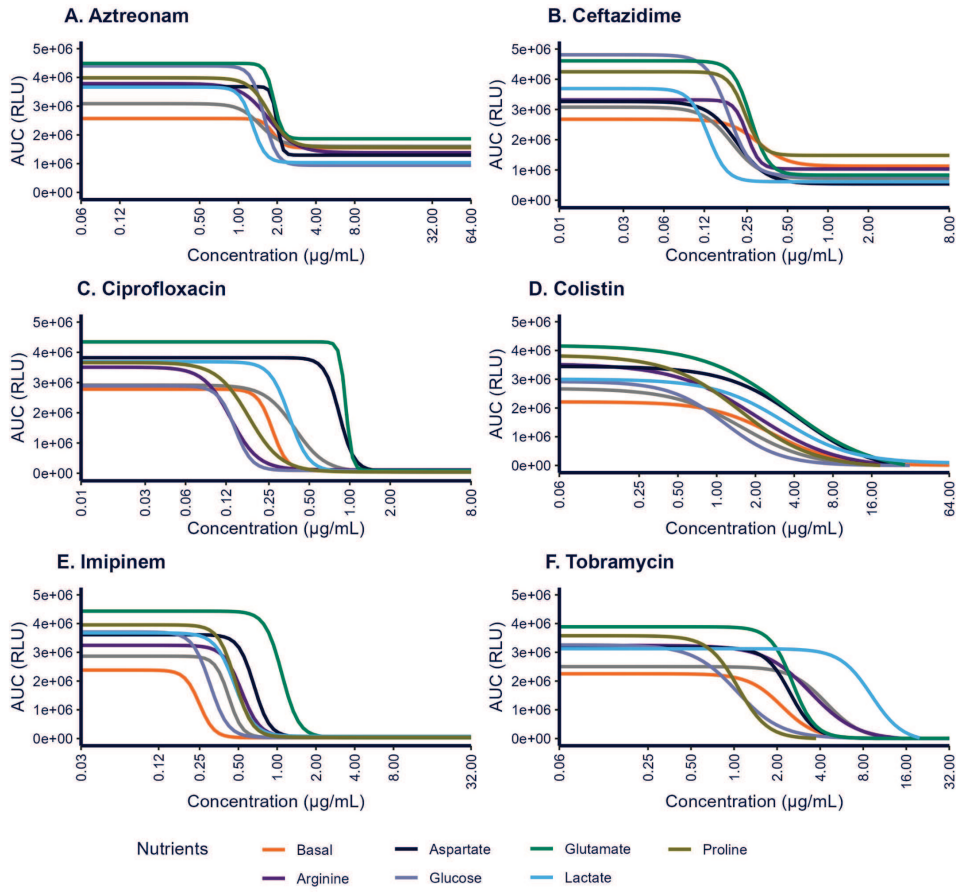

Figure 2. Pharmacodynamic exposure-response relationships for antibiotics cultured under different nutrient conditions**.** The area under the curve (AUC) for bacterial growth/kill based on relative light units (RLU) up to 15h in relation to antibiotic concentrations (n=9) were fitted using sigmoidal E_{max} curves, for different nutrient-enriched media formulations and the basal control media condition. The lines represent the mean predictions derived from 3 biological replicates (n = 3). Abbreviations: Aztreonam (AZT), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB).

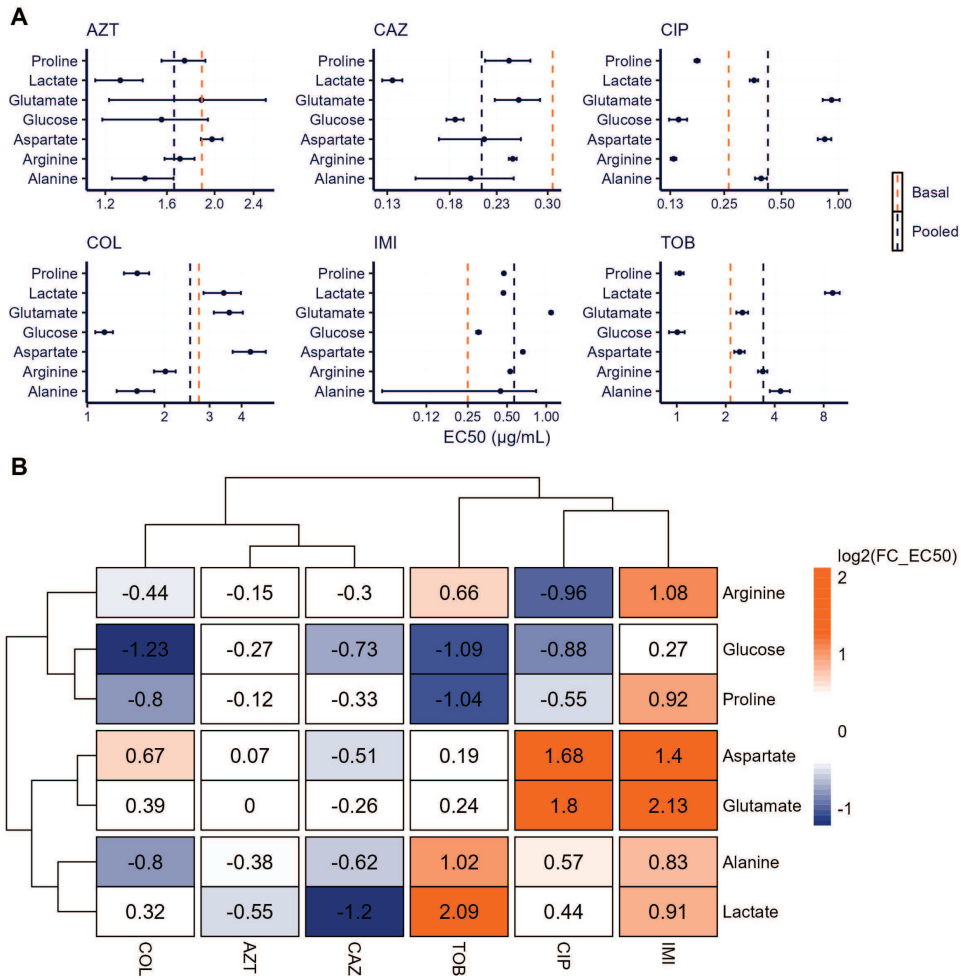

Figure 3. Changes in antibiotic sensitivity (EC_{50}) of P. aeruginosa across different nutrients and antibiotics. Observed area-under-the-curve for bacterial growth and kill for P. aeruginosa PAO1-Xen41 were regressed against drug concentrations for different antibiotics and nutrients, using a sigmoidal Emax function. The resulting EC_{50} estimates for different antibiotic-nutrient combinations are shown for (**A**) absolute EC50 values (mean and 95% confidence intervals), with vertical dashed lines indicating the EC50 obtained from the base media control treatment, and the cross-nutrient median EC_{50}, and (**B**) median fold-change (FC) values in EC_{50}, compared to the base media EC_{50}. The antibiotics and nutrients were clustered using Euclidean distance clustering to showcase patterns of antibiotic sensitivity and nutrient effect. Abbreviations: aztreonam (AZT), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB).

The relative EC_{50} would be the primary metric of relevance to quantitatively indicate subtle changes in drug potency, i.e., antibiotic sensitivity across conditions. For several nutrient conditions, we observed clinically relevant alterations in the EC_{50} values across different antibiotics (**Figure 3A**). We observed both reductions in EC_{50} as compared to the basal media and increased EC_{50} values, indicating increased resistance. Across all antibiotics, no clear trends in EC_{50} shifts were observed for specific nutrients.

When comparing the relative change in EC_{50} to the basal medium (**Figure 3B**), both aztreonam and ceftazidime exhibited similarly enhanced sensitivity across different nutrient conditions. The most notable changes were the increased sensitivity observed in lactate-enriched media for both antibiotics. In contrast, imipenem sensitivity was consistently reduced in all nutrient-enriched conditions, with the most significant reductions observed in aspartate- and glutamate-enriched media. For ciprofloxacin, colistin and tobramycin a wider variation in effect was compared to the basal medium. Glucose- and proline-enriched media resulted in a reduction of EC_{50}, while aspartate, glutamate- and lactate-enriched media increased the EC_{50} for all three antibiotics. The largest change in sensitivity was observed for tobramycin, where for lactate-rich media, the EC_{50} value increased profoundly (log2(FC_EC50) = 2.09, a 4.4-fold increase).

Fitness differences in different culture conditions affect PD parameters.

We studied the effect of different nutrient-enriched media under antibiotic-free conditions on fitness and growth yield using the growth curve profiles (**Figure 4**), to understand their potential contributions to differences in antibiotic response. Except for alanine, for all nutrients we found an increase of >1.5 fold in the upper limit of the model (E_0), i.e., the antibiotic baseline with no antimicrobial effect used in our pharmacodynamic analyses (**Figure S3**). To further understand these effects we calculated the maximum population growth rate (μ_{max}) and the maximum population density (N_{max}) of antibiotic-free conditions (**Figure S4**). While the nutrient composition significantly affected μmax, the magnitude of the effect was modest (**Figure 4B**), with an increase of up to 1.2-fold compared to the basal control media observed only for aspartate and glutamate. The observed effects on E_0 are predominantly explained by differences in N_{max} (**Figure 4C**), with a >2-fold increase observed for aspartate and glutamate and a fold change

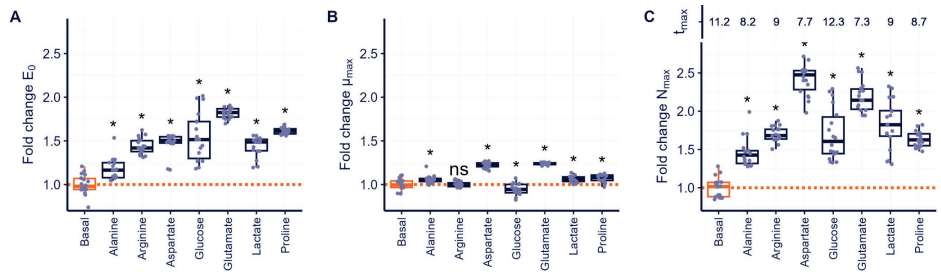

Figure 4. Nutrient effects on fitness and growth yield under antibiotic-free conditions. Growth curves for *P. aeruginosa* were analyzed for different media enriched for alanine, arginine, aspartate, glucose, glutamate, lactate, and proline on the fold change compared to basal media. (**A**) total growth yield described using the upper limit of the antibiotic concentration-response curve (E_0), (**B**) the maximal growth rate (μ_{max}) of the growth curve, (**C**) maximal population density (N_{max}) and the time (t_{max}) required to reach N_{max}. Significant changes compared to the basal control media are indicated using '*' for $p < 0.05$ and 'ns' for $p > 0.05$.

between 1.2 and 2.0 for all other nutrient conditions. Distinct differences in growth curves during the transition from the exponential growth phase to the stationary phase was visible (Figure S4), in particular for the time required to reach N_{max} (t_{max}).

The impact of differences in E_0 across different nutrient conditions on PD parameters was further evaluated by analyzing the total antimicrobial response. Comparing the relative EC_{50} with the absolute EC_{50} provides an indication of how the limits of PD model influence the total antimicrobial effect. The relative EC_{50} is defined as the midpoint between the two limits of concentration-response curve, whereas the absolute EC_{50} denotes a 50% reduction in the AUC from the baseline with no antimicrobial effect (E_0). A larger discrepancy between these EC_{50} values suggests a stronger impact of the two limits on determining the antibiotic EC_{50} [29]. For treatments with ciprofloxacin, colistin, imipenem and tobramycin, the difference between the average relative and absolute EC_{50} values was less than 5% (**Figure S5**). In contrast, ceftazidime and aztreonam treatments showed difference of respectively 14% and 22% indicating that differences in the PD model limits between the nutrient conditions do influence the determination of EC_{50}.

In vitro nutrient-driven PD differences impact treatment simulations with a clinically relevant tobramycin PK profile

To assess whether the magnitude of nutrient-associated changes in the PD response observed *in vitro* may have significance at clinically relevant antibiotic concentrations, we performed pharmacokinetic-pharmacodynamic (PK-PD) simulations. For proof of concept, we focused on tobramycin and the nutrients glucose and lactate, since for this antibiotic and these nutrient conditions clearly divergent PD effects were observed.

We re-fitted the PD model (**Equation 1**) with the in vitro obtained growth and kill rates from our luminescence time course data per antibiotic concentration. In the basal media enriched with glucose and lactate, maximum bacterial growth rates were similar (0.25 h^{-1} and 0.24 h^{-1}, respectively), as were the maximum bacterial kill rates (-0.15 h^{-1} and -0.14 h^{-1}, respectively) (**Figure S6**). However, the PD model estimated a 6-fold difference in the EC_{50} for glucose-enriched (1.4 µg/mL) and lactate-enriched (8.6 µg/mL) environments, indicating that tobramycin is profoundly more effective at lower concentrations in glucose-rich culture conditions.

We simulated clinical tobramycin concentration-time profiles using a previously published PK model for an intravenous dose of 3.3 mg/kg administered every 8 hours (**Figure 5A**). The tobramycin PK simulation shows that the free drug concentrations fell below the EC_{50} within 1 hour for glucose-rich conditions and within 5.5 hours for lactate-rich conditions after dose administration. As a result, treatment failure was observed for tobramycin under lactate-rich conditions, whereas growth suppression occurred in simulated glucose-enriched conditions (**Figure 5B**).

3.4. Discussion

In this study we used a combination of *in vitro* time-kill studies and mathematical modeling to investigate how specific nutrient conditions can distinctly affect bacterial growth and pharmacodynamic response of *P. aeruginosa* to different antibiotics.

We found that colistin, ciprofloxacin, imipenem and tobramycin demonstrated >2-fold differences in nutrient-dependent changes in antibiotic

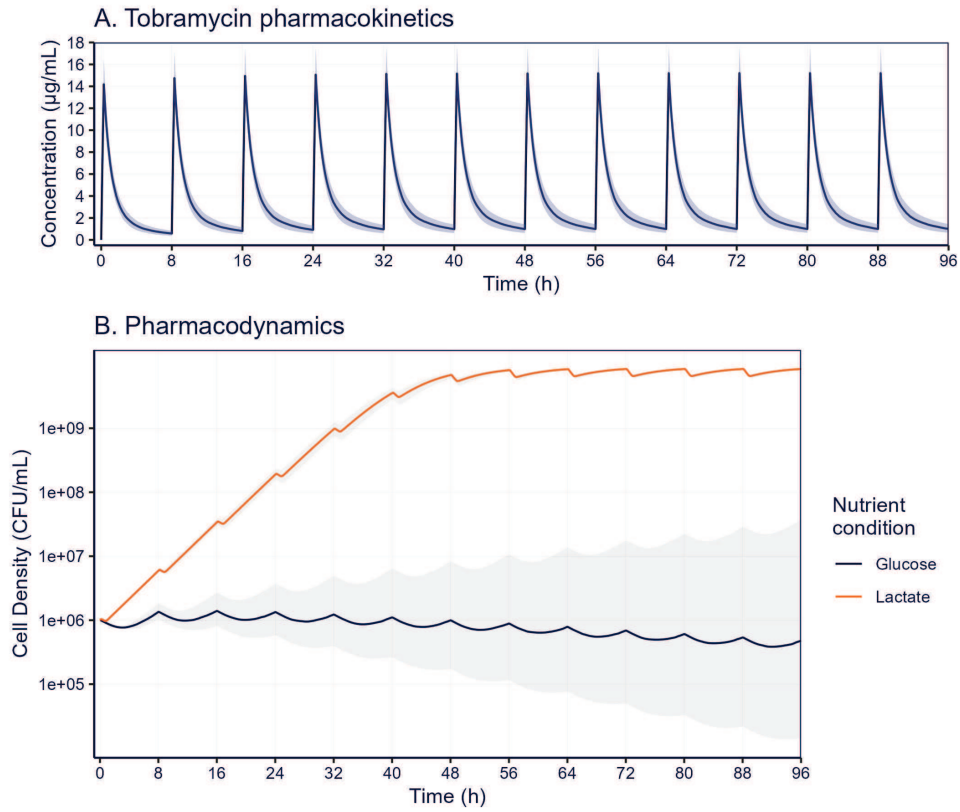

Figure 5. Pharmacokinetic and pharmacodynamic simulation of tobramycin treatment in glucose or lactate-rich environments. (**A**) Tobramycin concentrations are modelled using a two-compartment model following a 3.3 mg/kg q8h dosing regimen. (**B**) Treatment response is simulated using a pharmacodynamic model based on population growth rates per drug concentration from in vitro growth/kill curves. The solid lines represent the median (1000 simulations) with the interquartile range represented by the transparent-hued areas.

sensitivity (EC_{50}), while these nutrients only had a limited effect on changes in bacterial fitness. Our time-course analysis revealed that changes in growth dynamics induced by these antibiotics occur within the initial hours of treatment, even when nutrients are abundant and growth rates appear unchanged. This observation challenges the suggestion that antibiotic sensitivity changes were caused by nutrient depletion or diminished growth rates[30]. In contrast, the response to aztreonam and ceftazidime under various nutrient conditions was

more complex, as both the baseline response (E_0) and the maximum antimicrobial effect (E_{max}) were differently affected by the various nutrients.

Our findings indicate that the adding glucose to nutrient-limited media enhances colistin sensitivity. Variations in colistin sensitivity under different nutrient conditions are thought to arise from nutrient-induced changes in cell wall structure[14,15]. Glucose-rich conditions have been previously suggested to decrease colistin sensitivity by stabilizing intracellular osmotic pressure[14]. Our finding of enhanced colistin sensitivity thus challenges the hypothesis of osmotic stabilization of glucose in nutrient-scarce conditions. This observation is consistent with documented increases in colistin sensitivity in minimal media supplemented with glucose[31].

We found a diminished sensitivity of imipenem under nutrient conditions involving arginine, aspartate, glutamate, or proline. This can be explained by reduced imipenem uptake due to porin competition with these amino acids. Indeed, imipenem susceptibility in *P. aeruginosa* relies on the presence of outer membrane porins, particularly OprD and OprP, which facilitate the diffusion of sugars and amino acids[32–34]. Furthermore, nutrient starvation upregulates OprD[32,33,35], providing an explanation for the increased imipenem sensitivity observed in both basal and glucose-rich media. The reduced growth rate and short exponential growth phase in these conditions may prompt an earlier starvation response, thereby enhancing OprD-mediated imipenem uptake.

We observed reduced ciprofloxacin susceptibility in glutamate media, which has previously been associated with adaptations in nitrogen metabolism and stress responses[36,37]. This metabolic adaptation mitigates ciprofloxacin's antibacterial effect of inducing oxidative stress by increasing the generation of reactive oxygen species during oxidative phosphorylation[38,39]. The increased ciprofloxacin sensitivity observed in arginine-rich conditions may be attributed to the induction of biofilm formation during treatment. Arginine-induced biofilm formation imposes a high metabolic burden on the cells[40], aligning with the effective anti-biofilm activity of ciprofloxacin[41]. The difference in ciprofloxacin susceptibility among nutrient conditions might be due to a pH-dependent effect, although our medium was phosphate buffered to a pH of 7.4. Our observations in ciprofloxacin susceptibility correspond to previous findings of ciprofloxacin

being more effective in alkaline conditions, e.g. arginine, compared to less sensitivity in acidic conditions, e.g. glutamate and aspartate[42]. However, this pH-mediated effect is not present in the observed reduced tobramycin susceptibility in arginine-rich conditions. Unbuffered arginine increases media alkalinity, resulting in increased tobramycin cellular uptake by increasing the transmembrane potential[43].

In our study, for tobramycin, we observed enhanced sensitivity for proline and glucose, whereas for lactate and alanine, reduced sensitivity was found. So far previous studies have only investigated the effect of glucose-enriched media on *P. aeruginosa* tobramycin sensitivity, finding a similar potentiation effect[44,45]. Cellular respiration is key for aminoglycoside uptake, thereby directly relating tobramycin susceptibility to energy metabolism[44]. The nutrients alternated in our media compositions are all closely linked to the TCA cycle, and intermediate products have been consistently correlated with tobramycin potentiation[18,44-46]. Interestingly, the sensitivity enhancement associated with TCA cycle activity can be suppressed by reducing the production of electron carriers through the activation of pleiotropic metabolic pathways. The redox imbalance induced by these alternative pathways and anaerobic energy production can be mitigated through the utilization of lactate[47]. This observation may provide an explanation for the reduced susceptibility in lactate-rich media. Although proline and alanine demonstrated a profound effect on tobramycin treatment in our study, and previous research highlighted their role in alternative energy-producing pathways such as denitrification[48,49], their exact role in *P. aeruginosa* metabolism during tobramycin treatment remains to be investigated.

Our PK-PD simulation illustrates how differences in PD response under nutrient-enriched conditions may lead to clinically relevant changes in antibiotic treatment response. This is demonstrated using a clinical tobramycin PK profile and the PD parameters from glucose and lactate-enriched conditions. While these in vitro conditions do not fully replicate *in vivo* growth environments, which may also involve phenotypical adaptations such as biofilm formation or interspecies interactions, they underscore the relevance of considering nutrient conditions in the infectious microenvironment. This is especially relevant when nutrient availability could be altered under specific disease conditions. For instance, elevated lactate levels have been found in CF patients with declining

lung function[50], which could thus potentially contribute to the reduced tobramycin efficacy in adult CF patients[51]. Diabetes is a common disease in CF patients and for which increased glucose levels can be expected, which could potentially affect TOB treatment response[52].

The nutrient conditions employed in this study do not capture the full complexity of potential CF lung environments but provide isolated insights into the effect of specific nutrient conditions. Nutrients showed modest differential impact on bacterial fitness (μ_{max}) and profound changes in growth yield (N_{max}). The minimal impact on μ_{max} from substituting a single nutrient is consistent with prior studies on glucose and lactate addition to minimal media[53], and can be explained by a compressed nutrient utilization hierarchy under nutrient-poor conditions [54,55], facilitating the simultaneous utilization of the basal medium nutrients and the added nutrients. This efficient metabolic regulation of *P. aeruginosa* suggests that our findings may not directly extrapolate to other conditions or nutrient combinations. Future research, focusing specifically on nutrient utilization during antibiotic exposure, will be crucial to deepen our understanding of specific nutrients' roles in more complex environments.

In conclusion, our study demonstrates a profound impact of specific nutrient conditions on antibiotic sensitivity, with only modest effects on fitness. While broader clinical applicability of our results remains to be further elucidated, our work underscores the relevance of nutrients in the infectious microenvironment. Ultimately, it could be envisioned that specific nutrient levels in either plasma or sputum may be considered a clinically relevant predictor of antibiotic treatment response. Similarly, the effect of nutrient conditions may be important for consideration in antibiotic susceptibility testing.

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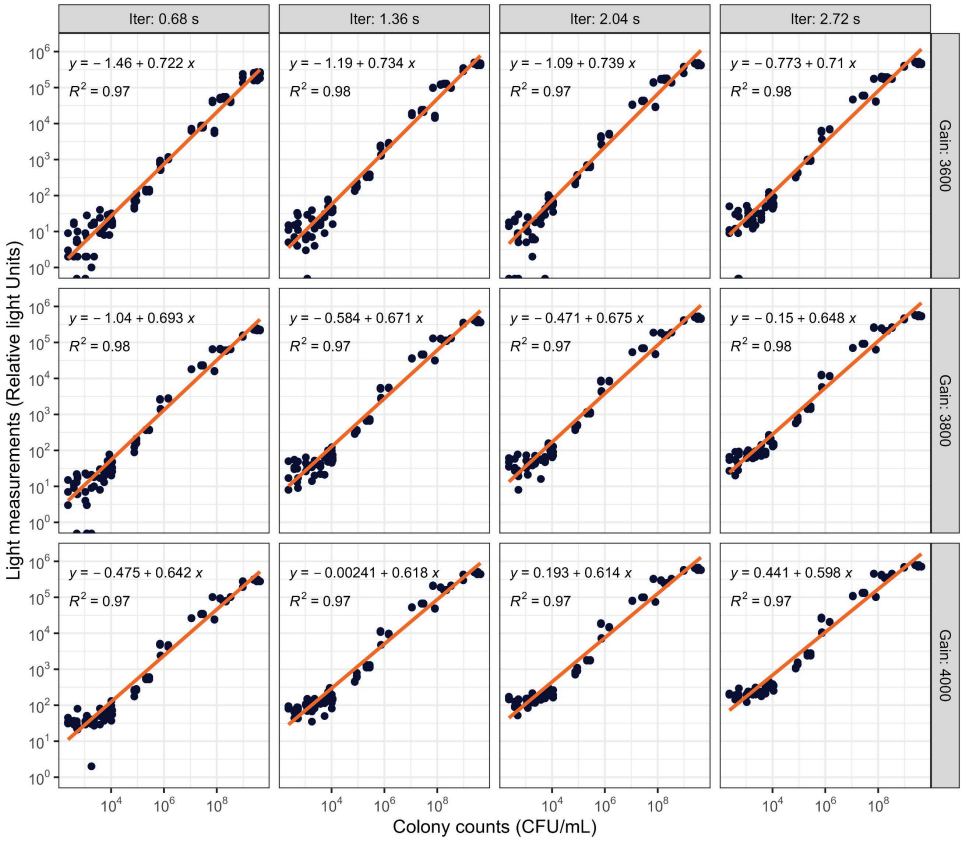
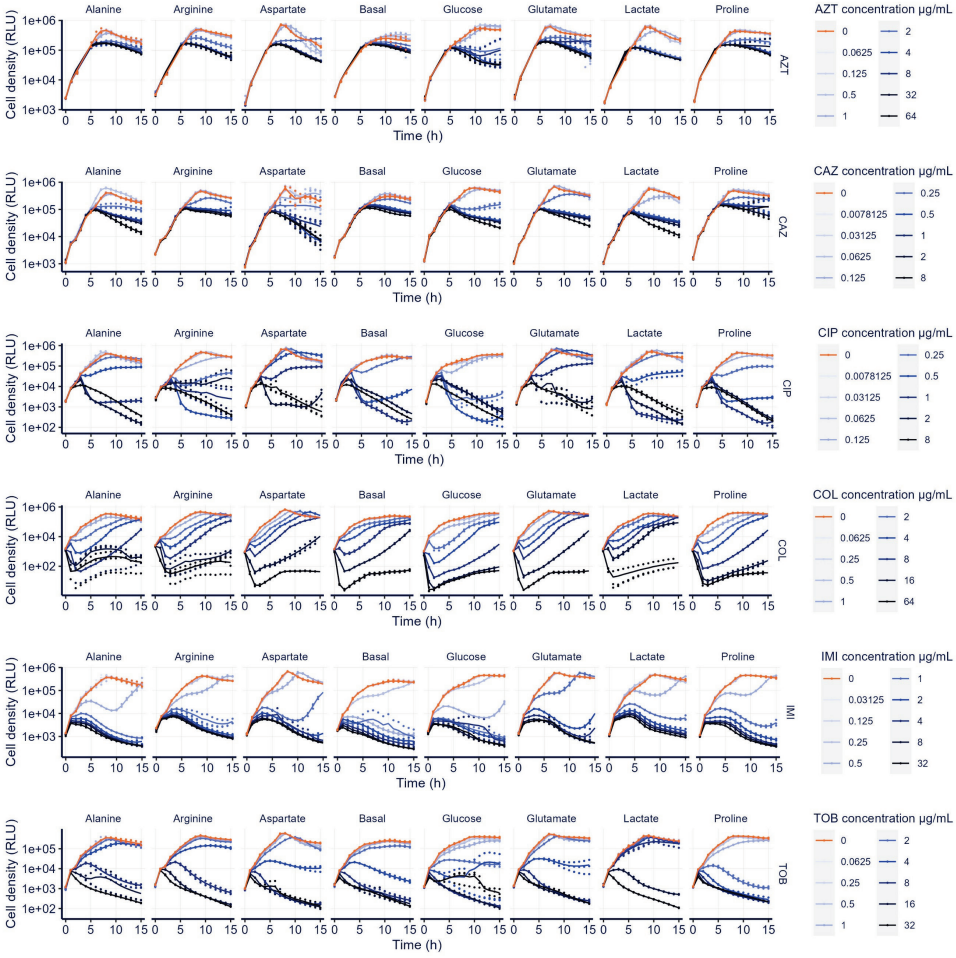
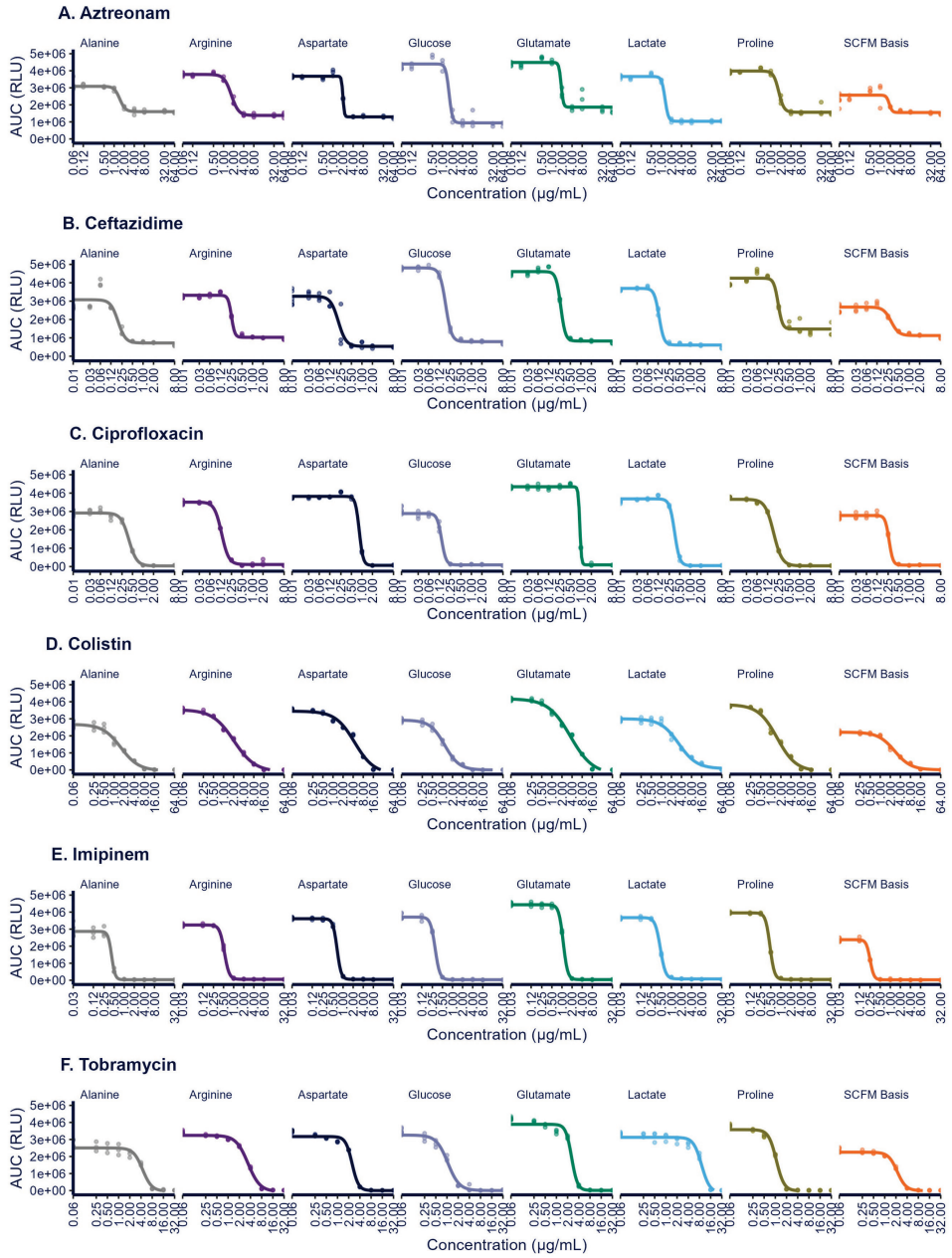
3.6. Supplementary figures and tables



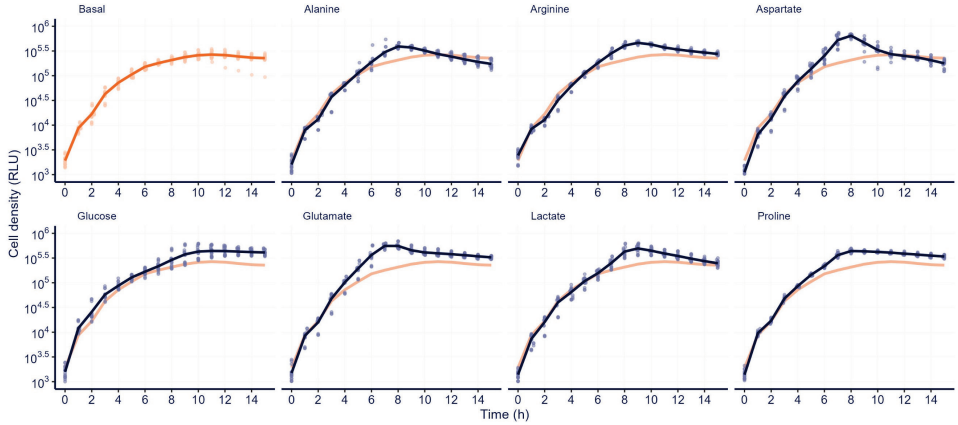
Supplemental Figure 1. Linear calibration between luminescence (relative light units, RLU) and cell counts (CFU/mL) for multiple combinations of detector settings, varying iteration time (iter, columns) and gain (rows). The iteration time stands for the total measurement time per well and the gain is amplification in the conversion from light into electric signal.



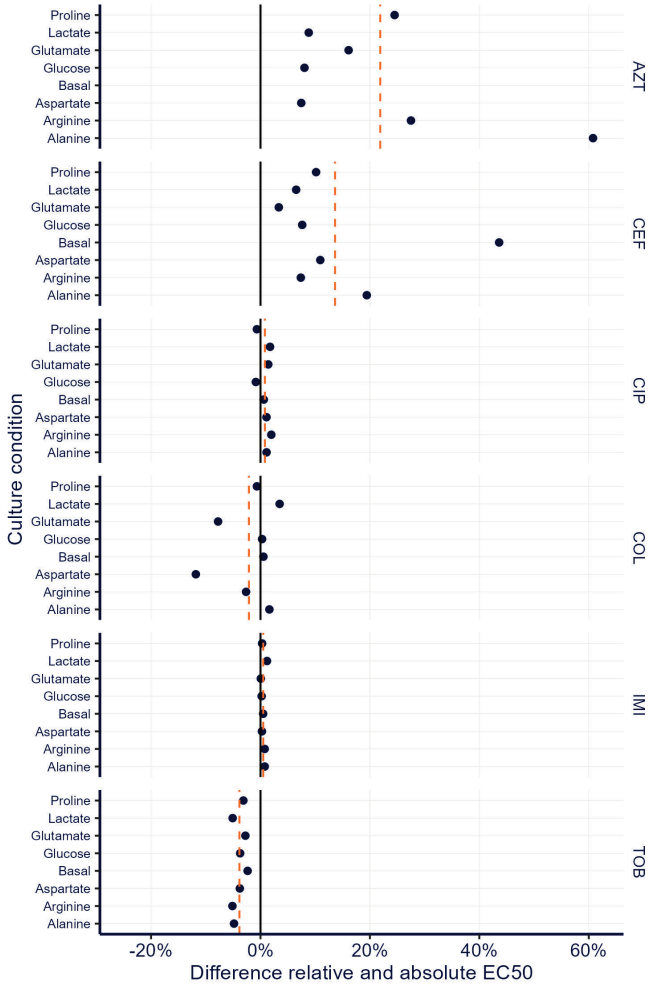
Supplemental figure 2. Dynamic analysis of the population size over time during the treatment of 6 antibiotics with 9 concentrations and a positive control in 8 media formulations. The y-axis is the cell density measured by relative light units (RLU). All conditions have 3 biological replicates. Abbreviations: aztreonam (AZT), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB).



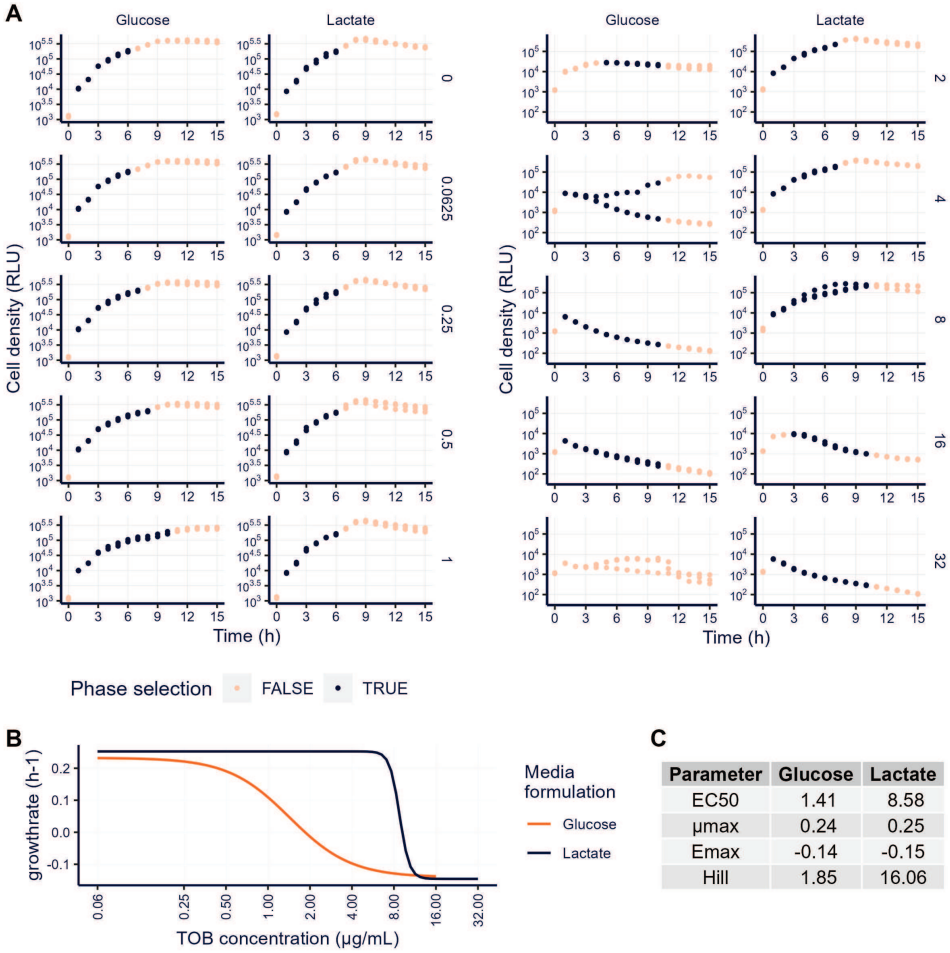
Supplemental Figure 3. Emax model fitting was performed on the area under the curve (AUC) of growth curves across varying antibiotic concentrations. The model was fitted using the average AUC values for each antibiotic concentration (n = 3). From this model, the upper limit (E0), the half-maximal effective concentration (EC50), and the lower limit (Emax) were determined.



Supplemental figure 4. The dynamic effect of the addition of nutrients (navy blue lines) to the basal (orange) media composition on the population size over 15-hours of incubation in antibiotic free conditions.



Supplemental figure 5. The difference between the relative half-maximal effective concentration (EC_{50}) and the absolute EC_{50}. The relative EC_{50} is extracted as the halve maximal response of the dose-response curve between the population fitness (E0) and the maximal drug effect (Emax). The absolute EC50 is extracted as the concentration at 50% reduction of E0. The difference between the two antibiotic sensitivity determinations is obtained by dividing the relative EC_{50} by the absolute EC_{50} concentrations per culture condition. Abbreviations: aztreonam (AZT), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB).



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Parameter	Glucose	Lactate
EC50	1.41	8.58
µmax	0.24	0.25
Emax	-0.14	-0.15
Hill	1.85	16.06

Supplemental figure 6. Phase selection for growth rate determination for growth rate based dose-response modeling of tobramycin (TOB). (**A**) The time-points included (blue dots) for the determination of the growth or kill rate of the tobramycin concentration using a linear regression. (**B**) The sigmoid E_{max} dose-response curve for glucose and lactate using the growth rate as response. (**C**) The pharmacodynamic parameters extracted from the dose-response model.

Supplemental table 1. Detailed content list of synthetic media

	Name	Concentration (mM)	Company information
M9 buffer	di-sodium hydrogen phosphate (Na_2HPO_4)	90.2	Thermo Fisher Scientific
	Potassium di-hydrogen phosphate (KH_2PO_4)	22.0	VWR International
	Sodium chloride (NaCl)	8.5	Merck KGaA (Avantor™)
	Ammonium chloride (NH_4Cl)	18.6	Alfa Aesar
	Magnesium sulphate hepta-hydrate ($MgSO_4$)	1.0	VWR International
	Calcium chloride ($CaCl_2$)	0.1	Acros Organics
Salts	Potassium nitrate (KNO_3)	0.35	Acros Organics
	Iron sulphate ($FeSO_4$)	0.0036	Alfa Aesar
Suppl.	BME Vitamin solution	*1x*	Thermo Fisher Scientific
Trace metals	Di-sodium Ethylene di-amine tetra-acetic acid (EDTA)	0.002 (mg/mL)	J.T. Baker (Avantor™)
	Zinc Sulphate hepta-hydrate ($ZnSO_4$)	0.23 (mg/mL)	Alfa Aesar
	Boric acid (H_3BO_3)	0.111 (mg/mL)	Acros Organics
	Manganese chloride tetra-hydrate ($MnCl_2$)	0.051 (mg/mL)	Sigma Aldrich (Avantor™)
	Cobalt chloride ($CoCl_2$)	0.017 (mg/mL)	Alfa Aesar
	Copper Sulphate penta-hydrate ($CuSO_4$)	0.015 (mg/mL)	Sigma Aldrich (Avantor™)
	Ammonium hepta-molybdate tetra hydrate ($(NH_4)_6\ Mo_7O_2$)	0.01 (mg/mL)	Alfa Aesar
Basis nutrients	Cysteine (Cys)	0.2	Chem-Impex International
	Glycine (Gly)	1.2	Acros Organics
	Histidine hydrochloride (His)	0.5	Chem-Impex International
	Isoleucine (Ile)	1.1	Chem-Impex International
	Leucine (Leu)	1.6	Chem-Impex International
	Lysine hydrochloride (Lys)	2.1	Thermo Fisher Scientific
	Methionine (Met)	0.6	Chem-Impex International
	Phenylalanine (Phe)	0.5	Chem-Impex International
	Serine (Ser)	1.4	Chem-Impex International
	Threonine (Thr)	1.0	Chem-Impex International
	Tryptophan (Trp)	0.01	Chem-Impex International
	Tyrosine (Tyr)	0.8	Chem-Impex International
	Valine (Val)	1.1	Chem-Impex International
Nutrient alterations	Alanine (Ala)	15	Chem-Impex International
	Arginine (Arg)	15	Chem-Impex International
	Aspartate (Asp)	15	Chem-Impex International
	Glutamate (Glu)	15	Chem-Impex International
	Sodium lactate (LAC)	15	Biosynth International
	Proline (Pro)	15	Thermo Fisher Scientific
	Glucose (GLC)	15	Alfa Aesar

Supplemental table 2. Pharmacokinetic parameters

Explanation	Name	Value / Formula	Unit
Patient bodyweight	BW	55.3	kg
Patient age		29.0	years
Clearance rate per BW	CL_t	0.1212	L/h/kg
Volume comp. 1 per BW	V_C	0.20	L/kg
Distribution rate per BW	CL_d	0.0702	L/h/kg
Volume comp. 2 per BW	V_{ss}	0.38	L/kg
Individual Variability (η)	η_{CL_t} η_{V_C} η_{CL_d} $\eta_{V_{ss}}$	28.5 28.2 66.6 27.8	% % % %
Population size		1000	
Dosing interval		8	h
Dosing amount		3.3 * BW	mg
Dosing duration		0.30	h
Volume compartment 1	$V_{central}$	$V_C * e^{\{iv\}} * BW$	L
Elimination rate from $V_{central}$	$k_{elimination}$	$(CL_t * e^{\{iv\}} * BW) / V_{central}$	
Volume compartment 2	V_2	$V_{ss} * e^{\{iv\}} * BW$	L
Rate constant 1-->2	K_{12}	$(CL_d * e^{\{iv\}} * BW) / V_{central}$	
Rate constant 2-->1	K_{21}	$(CL_d * e^{\{iv\}} * BW) / V_2$	
Amount in compartment 1	$m_{central}$	$\frac{m_{central}(t)}{dt} = k_{21} * m_2 - (k_{elimination} + k_{12}) * m_{central}$	mg
Amount in compartment 2	m_2	$\frac{m_2(t)}{dt} = K_{12} * m_{central}(t) - k_{21} * m_2(t)$	mg
Concentration compartment 1	$C_{central}$	$m_{central} / V_{central}$	mg/L

Explanations	Name	Value / Formula		Unit
		Glucose	Lactate	
Max. drug effect	E_{max}	-0.144	-0.146	h^{-1}
Max. growth rate	K_{growth} (E_0)	0.240	0.254	h^{-1}
Half effective concentration	EC_{50}	1.406	8.582	mg/L
Hill coefficicent	n_H	1.850	16.057	
Starting population	N_0	$1 * 10^6$		CFU/mL
Max. population	N_{max}	$9 * 10^9$		CFU/mL
Effective growth rate	k_{effect}	$K_{growth} - (E_{max} + \frac{K_{growth} - E_{max}}{1+e^{n_H(\log(C_{central}) - \log(EC_{50}))}})$		h^{-1}
Infection population	N(t)	$\frac{dN(t)}{dt} = (k_{growth} * (1\text{-}N(t)/N_{max}) - k_{effect}) * N(t)$		CFU/mL

Supplemental table 3. Pharmacodynamic parameters

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 4

Prolonged anoxic exposure impacts antibiotic sensitivity profiles of *Pseudomonas aeruginosa*

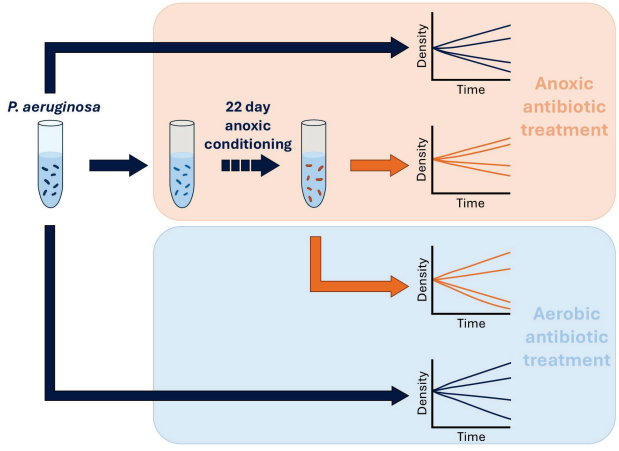
Maik Kok, Wisse van Os, Thomas Hankemeier, Coen van Hasselt

FEMS Microbiology Letters, 2025, 372, fnaf066

Abstract

Chronic respiratory tract infections with *Pseudomonas aeruginosa* frequently occur in patients with cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis. A hallmark of these conditions is the accumulation of mucus plugs, creating oxygen-limited niches. Within these microenvironments, *P. aeruginosa* undergoes cellular modifications that may alter its antibiotic sensitivity. Although the acute effects of anoxia are well studied, the impact of prolonged anoxic exposure on antibiotic sensitivity remains unclear. In this study, we developed anoxic-conditioned *P. aeruginosa* strains by passaging a laboratory strain for 22 days in an anoxic environment. We performed time-kill assays with both parental and anoxic-conditioned strains in anoxic and aerobic environments, using ceftazidime, ciprofloxacin, colistin, and tobramycin. The anoxic-conditioned strains exhibited increased susceptibility to tobramycin and reduced sensitivity to colistin and ceftazidime. These differences were attributed to altered killing rates (as with tobramycin) or reduced regrowth under anoxic conditions (as with colistin). For ciprofloxacin, a steeper killing rate was observed against the anoxic-conditioned strains, but 24-hour outcomes were similar to the parental strain. Overall, our findings demonstrate that long-term anoxia alters antibiotic sensitivity in *P. aeruginosa* differently than acute anoxia, with important implications for treating chronic infections in oxygen-limited environments.

Graphical abstract



4.1. Introduction

Lung diseases such as chronic lung conditions such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and non-CF bronchiectasis[1,2] are characterized by anoxic environments. These anoxic environments are caused by airway narrowing and the buildup of thick, dehydrated mucus limit oxygen penetration, and high oxygen consumption of the host immune response further exacerbates anoxia[3]. In these anoxic niches, the respiratory pathogen *Pseudomonas aeruginosa* uses fermentation and alternative electron acceptors to sustain cellular functions and proliferation[4,5]. These metabolic adaptations that support survival of P. aeruginosa in anoxic niches can also influence its sensitivity to antibiotics. Antibiotics commonly used against *P. aeruginosa*, including tobramycin, ciprofloxacin, and ceftazidime, rely on oxygen-dependent processes, such as oxidative phosphorylation, reactive oxygen species production, and active bacterial proliferation to exert their antimicrobial effects[6]. In contrast, colistin remains effective in anoxic environments because energy-starved cells are unable to modify their membranes to reduce colistin binding[7].

While the differential impact of acute responses to anoxic conditions on antibiotic sensitivity have been previously studied, the consequences of prolonged anoxia-induced adaptations remain largely unknown. Longer-term genetic or transient changes in cellular metabolism, stress responses, biofilm formation, and membrane remodeling have yet to be characterized in detail[8–10]. Alterations in membrane composition and transport proteins may affect the uptake and effect of membrane-targeting antibiotics such as ceftazidime and colistin. Additionally, metabolic specialization to anoxia introduces cellular redox imbalances that can alter antibiotic activity[11]. In parallel, the development of oxygen intolerance due to anoxic specialization can lead to higher levels of reactive oxygen species (ROS) upon re-exposure to oxygen[12]. These prolonged metabolic adaptations can influence the sensitivity of ROS-dependent antibiotics such as ciprofloxacin and metabolism-dependent antibiotics such as tobramycin. Thus, long-term anoxic adaptation affects key mechanisms relevant to antibiotic activity, but the potential impact on antibiotic sensitivity remains unclear.

In this study, we investigated how prolonged anoxic exposure affects the sensitivity of P. aeruginosa to ceftazidime, ciprofloxacin, colistin and tobramycin. An anoxic-conditioned strain was obtained by passaging a P. aeruginosa laboratory strain in an anoxic environment (<1% oxygen) for 22 days. The effects of the antibiotic-classes were then compared between the conditioned and parental strains under both anoxic and atmospheric oxygen conditions using time-kill assays, providing a comprehensive view of how anoxic specialization influences antibiotic sensitivity.

4.2. Materials and Methods

Strains, culture media, agar plates, and antibiotics

Pseudomonas aeruginosa PAO1 (DSM1117) was used as the parental strain in this study. Synthetic cystic fibrosis sputum medium (SCFM) served as the liquid medium for anoxic conditioning and time-kill experiments (**Table S1**)[13]. Samples for colony-forming unit (CFU) enumeration were diluted using a 1:4 dilution of SCFM with 0.11 M phosphate buffer before plating. Agar plates were prepared with Mueller-Hinton broth (MHB) agar supplemented with 10 mM KNO_3 (Acros Organics, Geel, Belgium) to prevent the loss of oxygen-intolerant populations during aerobic plating.

Antibiotic solutions were prepared and diluted with SCFM to the desired concentrations in microtiter plates using an Opentrons OT-2 liquid handling system (Opentrons Inc., New York, NY, USA) one day before the time-kill assay. Ceftazidime pentahydrate was purchased from Thermo Fisher Scientific (Breda, The Netherlands), ciprofloxacin and tobramycin from Chem-Impex International (Wood Dale, IL, USA), and colistin sulfate from Cayman Chemical Company (Ann Arbor, MI, USA).

Anoxic culture environment and anoxic conditioning

Anoxic cultures were performed in a Baker Ruskin anoxic workstation (Sanford, Maine, United States). All liquid media used for anoxic experiments were pre-conditioned to the anoxic environment (<1% oxygen) for two days, and antibiotic microtiter plates were placed in the anoxic chamber one day before the time-kill assay.

Anoxic-conditioned strains were generated in triplicate, each from randomly selecting colonies of the parental strain grown overnight on an aerobic agar plate culture. Conditioning was conducted in triplicate using 2 mL pre-acclimated SCFM medium in sealed 12 mL culture tubes at 37 °C in the anoxic chamber. Every two days, 20 µL of the cultures was transferred into fresh SCFM, continuing for 22 days to achieve anoxic conditioning.

Time-kill assay

Time-kill assays in SCFM were performed in triplicate for the parental and anoxic-conditioned *P. aeruginosa* strains in aerobic and anoxic environments to examine antibiotic sensitivity (**Figure S1**). The parental strain replicates were subcultured for 20 hours under aerobic conditions, while the anoxic conditioned strains were used directly following the 22-day incubation period. Liquid cultures from the parental and anoxic-conditioned strain were diluted to reach a starting density of approximately 1×10^7 CFU/mL, based on optical density measurements at 600 nm. Cultures were exposed to five antibiotic concentrations in 2-fold serial dilutions, along with an antibiotic-free control. Antibiotic concentrations for the assays were guided by visual MIC testing of the parental strain performed aerobically (**Table S2**). MIC determination under anoxic conditions was not feasible due to low final culture densities.

Anoxic time-kill assays were incubated within the anoxic chamber, while aerobic assays were incubated at 37 °C with shaking (250 rpm) to ensure oxygenation. At 2 and 24 hours, 100 µL samples were taken, subjected to 10-fold serial dilutions, and plated on agar. All agar plates were incubated under aerobic conditions at 37 °C for one day, followed by an additional day at room temperature, before colony counting to determine bacterial population size.

Growth rate assay

Growth curves were obtained in quadruplicate in both aerobic and anoxic culture environments for the anoxic-conditioned and parental *P. aeruginosa* strains to compare antibiotic-free growth kinetics. Growth curves in the aerobic environment were obtained by transferring microtiter plates every 30 minutes between a Liconic StoreX STX44 incubator (Mauren, Liechtenstein) shaking at 150 rpm and a BMG Labtech Fluostar Omega microplate reader (Ortenberg, Germany), using a Peak Analysis and Automation KX-2 laboratory robot

(Hampshire, United Kingdom). Growth in the anoxic environment was measured every 30 minutes using a wireless Cerillo Alto plate reader (Charlottesville, VA, United States).

Data processing

Drug effects were quantified by calculating the change in bacterial population size over 24 hours of antibiotic exposure. To evaluate the initial bacterial response, the antibiotic net killing rate during the first 2 hours was determined by calculating the slope using the logarithmically transformed population sizes (N) and the elapsed time (Δt) (**Equation 1**). Maximal growth rates in antibiotic-free media were calculated using the splines function from the *growthrates* package in R, based on the growth curves from four technical replicates. All data analysis was performed in R (version 4.3.0)

$$Killing\ rate = \frac{log_{10}(N_2) - log_{10}(N_1)}{\Delta t} \quad (1)$$

4.3. Results

Acute and prolonged anoxic exposures modulate antibiotic effect over 24 hours

We investigated how anoxic conditions affect antibiotic activity by comparing time-kill curves obtained in aerobic and anoxic environments, both for the parental and the anoxic-conditioned strains. Time-kill experiments with the parental strain were performed to evaluate the acute effects of anoxic environments, whereas experiments with the anoxic-conditioned strain reflected prolonged adaptation to anoxia. We compared the change in bacterial population size between the inoculum and the 24-hour timepoint as a measure of antibiotic effect (**Figure 1**).

For ciprofloxacin, population size reduction of the parental strain was observed at 0.031 mg L^{-1} in anoxic environments, while 0.125 mg L^{-1} was required in aerobic environments. The anoxic-conditioned strain showed a similar oxygen-dependent response to ciprofloxacin but at one serial-dilution step lower (0.016 mg L^{-1} in anoxic and 0.062 mg L^{-1} in aerobic environments).

With tobramycin, population sizes of the parental strain started to decline after 24 hours at concentrations above 1 mg L^{-1}, with a notably more

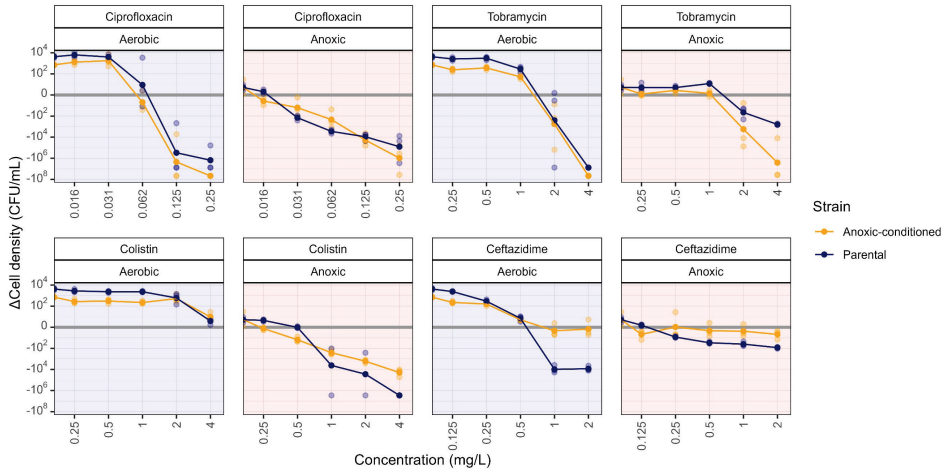


Figure 1. Change in cell density of the parental and anoxic-conditioned *P. aeruginosa* strains after 24 hours of antibiotic exposure in aerobic and anoxic environments. Orange lines and points represent the anoxic-conditioned *P. aeruginosa* strain, evolved over 22 days in an anoxic environment prior to antibiotic exposure, while navy lines and points represent the parental PAO1 strain. Solid lines and points denote the mean $\log_{10}$ change in cell density, calculated from the three biological replicates, which are represented as translucent points.

pronounced reduction at 4 mg L^{-1} under aerobic compared to anoxic conditions. At these concentrations, the anoxic-conditioned strain mirrored the aerobic response of the parental strain in both oxygen environments.

For both strains, all evaluated colistin concentrations failed to induce net reductions in population size in aerobic environments. However, reductions were observed in anoxic environments, with a smaller reduction for the anoxic-conditioned strain.

For ceftazidime, a flat concentration–effect relationship was observed in anoxic environments for both strains. In the aerobic environment, on the other hand, a clear concentration–effect relationship was observed for the parental strain, with population size reductions above 1 mg L^{-1}. In contrast, the highest tested ceftazidime concentration failed to reduce the population size of the conditioned strain in the aerobic environment.

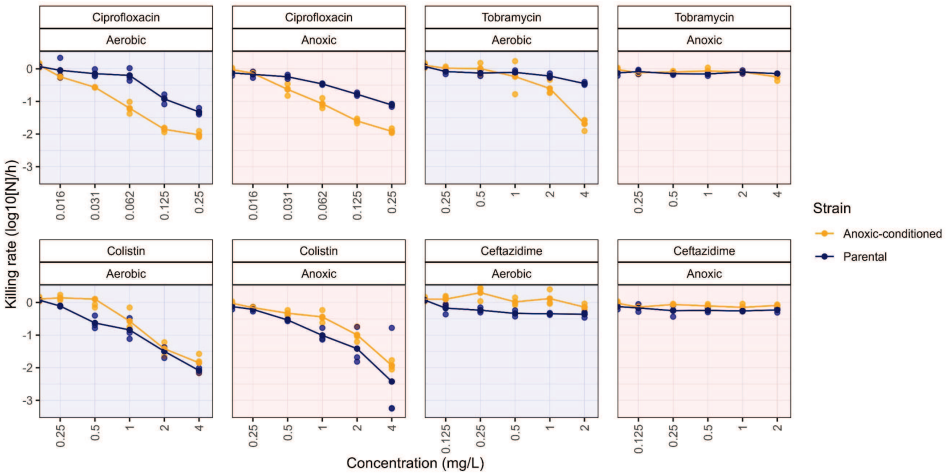


Figure 2. Net antibiotic killing rates in parental and anoxic-conditioned *P. aeruginosa* under aerobic and anoxic environments. Net killing rates during the initial 2 hours of antibiotic exposure for each biological replicate (shown as translucent points) were calculated by determining the slope of the change in $\log_{10}$-transformed cell density. Solid orange lines and points represent the mean response rate of the conditioned P. aeruginosa strain, which was evolved in an anoxic environment for 22 days prior to treatment, while navy represents the mean response rate of the parental PAO1 strain.

Anoxic conditioning impacts antibiotic net killing rates

To assess the effect of anoxic conditioning on the immediate bacterial response to antibiotics, we analyzed net killing rates during the first 2 hours of antibiotic exposure (**Figure 2**). The net killing rate was defined as the slope of the $\log_{10}$-transformed bacterial population size over time ($\log_{10}[\Delta N]/t$), with steeper negative slopes indicating faster killing. For ciprofloxacin, faster killing rates were observed at concentrations of 0.031 mg L^{-1} and above for the anoxic-conditioned strain compared to the parental strain, in both aerobic and anoxic culture environments. Tobramycin demonstrated consistent effects across most conditions, except for the anoxic-conditioned strain in aerobic environments, for which the fastest killing rate of -1.68 (corresponding to a 50-fold reduction per hour) was observed at 4 mg L^{-1}, compared to -0.45 for the parental strain (2.8-fold reduction). For colistin, similar killing rates between strains and oxygen environments were observed, with rates becoming more negative at higher concentrations. The largest magnitude of killing (-2.42, corresponding to a 263-

fold reduction per hour) was observed at 4 mg L^{-1} in the parental strain under anoxic conditions. By contrast, ceftazidime had minimal killing across all strains and environments, with a fastest killing rate of -0.36 (a 2.3-fold reduction per hour).

Prolonged anoxic exposure reduces bacterial growth rates

To assess fitness differences between the parental strain and anoxic-conditioned strains, we derived the maximal growth rates under antibiotic-free conditions. Here, the growth rate reflects the time a strain requires for doubling its population size. The growth rate of the parental strain was on average 4.9-fold increase in aerobic than in anoxic environments, and for the anoxic-conditioned strain it was 8.1-fold increased (**Figure 3**). A moderate increase in growth rate was observed for the parental strain than for the anoxic-conditioned strain in both aerobic (0.63 h^{-1} vs. 0.57 h^{-1}) and anoxic (0.13 h^{-1} vs. 0.07 h^{-1}) environments.

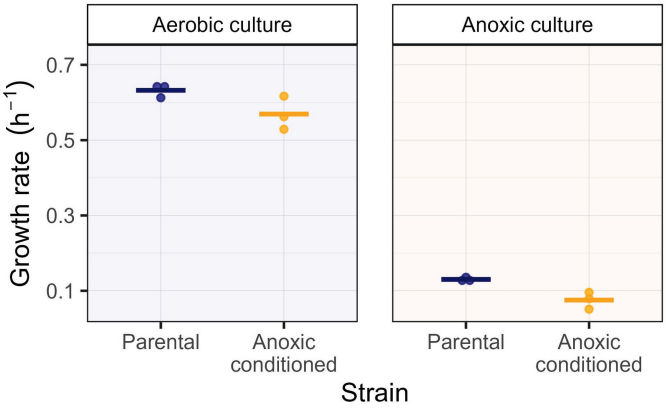


Figure 3. Growth rates of the parental and anoxic-conditioned *P. aeruginosa* strains in aerobic and anoxic environments. Maximal growth rates were calculated from smoothed spline growth curves of four technical replicates per strain and condition. The growth curve is based on optical density at 600 nm (OD600) measurements taken every 30 minutes. Data points represent µmax values per biological replicate, and the solid line indicates the mean µmax of the three biological replicates.

4.4. Discussion and conclusions

In this study, we demonstrated how prolonged adaptation of a *P. aeruginosa* laboratory strain to anoxic environments reduces bacterial growth rates and alters antibiotic susceptibility in a class-dependent manner. Anoxic specialization resulted in increased effect of tobramycin, reduced effects of colistin and ceftazidime, and a higher initial killing rate for ciprofloxacin. In the aerobic environment, ciprofloxacin and tobramycin exhibited increased initial killing rates against the anoxic-conditioned strain whereas ceftazidime effects after 24 hours of exposure were decreased.

The limited difference in ciprofloxacin effects over 24 hours exposure between anoxic and aerobic environments aligns with previous findings that oxygen-deprived environments primarily induce fluoroquinolone tolerance rather than altering antibiotic susceptibility[14,15]. The steeper killing rate observed in the anoxic-conditioned strain under aerobic conditions is consistent with the dependence of fluoroquinolones on reactive oxygen species for activity[16]. Oxygen radicals are formed in greater quantities when oxygen is introduced to cells that have undergone anoxic specialization[12]. The increased ciprofloxacin effect observed in the anoxic-conditioned strain in the anoxic environment suggests that additional biological adaptations to anoxia may influence fluoroquinolone effects.

Anoxic conditioning increased tobramycin sensitivity in both aerobic and anoxic environments, contrasting with the established reliance of aminoglycosides on the proton motive force for intracellular entry[17]. Although oxygen-deprived environments typically exhibit lower membrane potentials, active transport systems can still facilitate aminoglycoside uptake[18]. Once inside, aminoglycosides trigger secondary stress responses, such as envelope and oxidative stress, potentially explaining the enhanced 24-hour tobramycin effect on the anoxic-conditioned strain. Despite similar 24-hour outcomes between the strains in the aerobic environment, a substantially higher killing rate was observed in the anoxic-conditioned strain compared to the parental strain, highlighting the impact of prolonged anoxic adaptation on aminoglycoside activity. These observations align with reports of pronounced differences in

aminoglycoside sensitivity in clinical CF respiratory tract isolates across oxygen gradients, likely due to prior exposure to anoxic environments[14,15,19].

The drug effect of colistin after 24 hours of exposure was stronger in the anoxic environment for both strains, consistent with previous findings that oxygen deprivation enhances colistin activity [20,21]. The increased net effect in anoxic conditions may be due to lower bacterial growth rates in this environment, which can result in higher net effect or reduced regrowth. Notably, the killing rates did not substantially differ between oxygen conditions, and the anoxically conditioned strain showed a reduced colistin effect. This suggest a more complex interplay between colistin's mechanism of action and *P. aeruginosa* responses under varying oxygen levels. *P. aeruginosa* is known to modify the lipopolysaccharide composition of its membrane[22], the primary target of colistin [23], as a resistance mechanism when energy supplies are sufficient[7]. This may explain the regrowth observed in an aerobic culture environment. Currently, nebulized colistin is extensively evaluated as an adjunctive therapy to prevent ventilator-associated pneumonia (VAP) in mechanically ventilated patients[24]. Our results suggest that increased lung oxygenation through ventilation might negatively affect colistin efficacy, potentially contributing to the limited effectiveness of prophylactic colistin treatment in reducing VAP incidence[25]. Colistin exhibits greater clinical efficacy in CF respiratory infections, where anoxic microenvironments are common[26]. However, the role of anoxia in lipopolysaccharide modification remains poorly understood, such modifications may also explain the reduced colistin effects against the anoxic-conditioned strain. Investigating these modifications further represents an important direction for future research.

Ceftazidime displayed a distinct pharmacodynamic profile compared to the other antibiotics tested. No differences in killing rates were observed during the initial hours of exposure, which is consistent with the time-dependent activity of ceftazidime[27]. However, over the full 24-hour period, ceftazidime sensitivity was markedly reduced following anoxic conditioning, both under aerobic and anoxic assay environments. Although ceftazidime is typically bactericidal for *P. aeruginosa*[28], this effect was only evident in the parental strain, albeit to a lesser degree in anoxic conditions. For the anoxic-conditioned strains, the effect of ceftazidime was limited to bacteriostasis in both aerobic and anoxic environments. This reduction in activity may be explained by previous work

showing that oxygen deprivation enhances efflux pump activity[29]. Reduced ceftazidime sensitivity in anaerobic biofilms has been reported[30], and our results demonstrate that this effect persists even after re-culturing under aerobic conditions.

We adopted a 22-day adaptation period to induce anoxic specialization. We compared the anoxically conditioned strain directly to the unpassaged parental strain, rather than to a 22-day aerobically subcultured strain. This choice was deliberate, as prolonged aerobic propagation would introduce substantially different genetic drift due to markedly higher growth rates and cell densities compared to the anoxic culture environment. While we acknowledge the limitations of this control setup, it allowed us to more accurately attribute observed differences to direct anoxic adaptation resulting from prolonged conditioning. Additionally, we recognize that the 22-day anoxic incubation used here is considerably shorter than typically experienced by *P. aeruginosa* strains persistently present in the CF lung, where prolonged evolutionary pressures may drive further specialization[31]. The current study utilized *P. aeruginosa* PAO1 laboratory strain to specifically study the effects of anoxic specialization on antibiotic response. Clinical isolates exhibit diverse adaptations due to prolonged in-host evolution, potentially affecting responses to anoxic environments. Using a defined laboratory strain ensures consistent genetic and phenotypic backgrounds, facilitating clearer interpretations of the observed antibiotic responses. Although beyond the scope of this study, future research involving clinical isolates represents a logical and necessary next step. We anticipate that such studies will require larger sample sizes to address the inherent heterogeneity among isolates, and they will greatly benefit from the experimental framework and treatment conditions established here. Finally, investigations into membrane modifications and redox imbalances could yield deeper mechanistic insights to inform the optimization of treatment strategies.

In conclusion, our findings show that anoxic adaptation of a *P. aeruginosa* laboratory strain modifies the effects of ceftazidime, ciprofloxacin, colistin, and tobramycin differently compared to acute anoxic exposure of the parental strain. Ciprofloxacin and tobramycin became more effective, whereas colistin and ceftazidime exhibited reduced effects against the anoxic-conditioned strain.

These altered antibiotic effects were also observed under aerobic conditions, suggesting sustained anoxia-induced cellular adaptations that alter antibiotic sensitivity. These findings highlight the importance of considering oxygen gradients in research aimed at optimizing antibiotic treatment for chronic *P. aeruginosa* infections in the CF lung.

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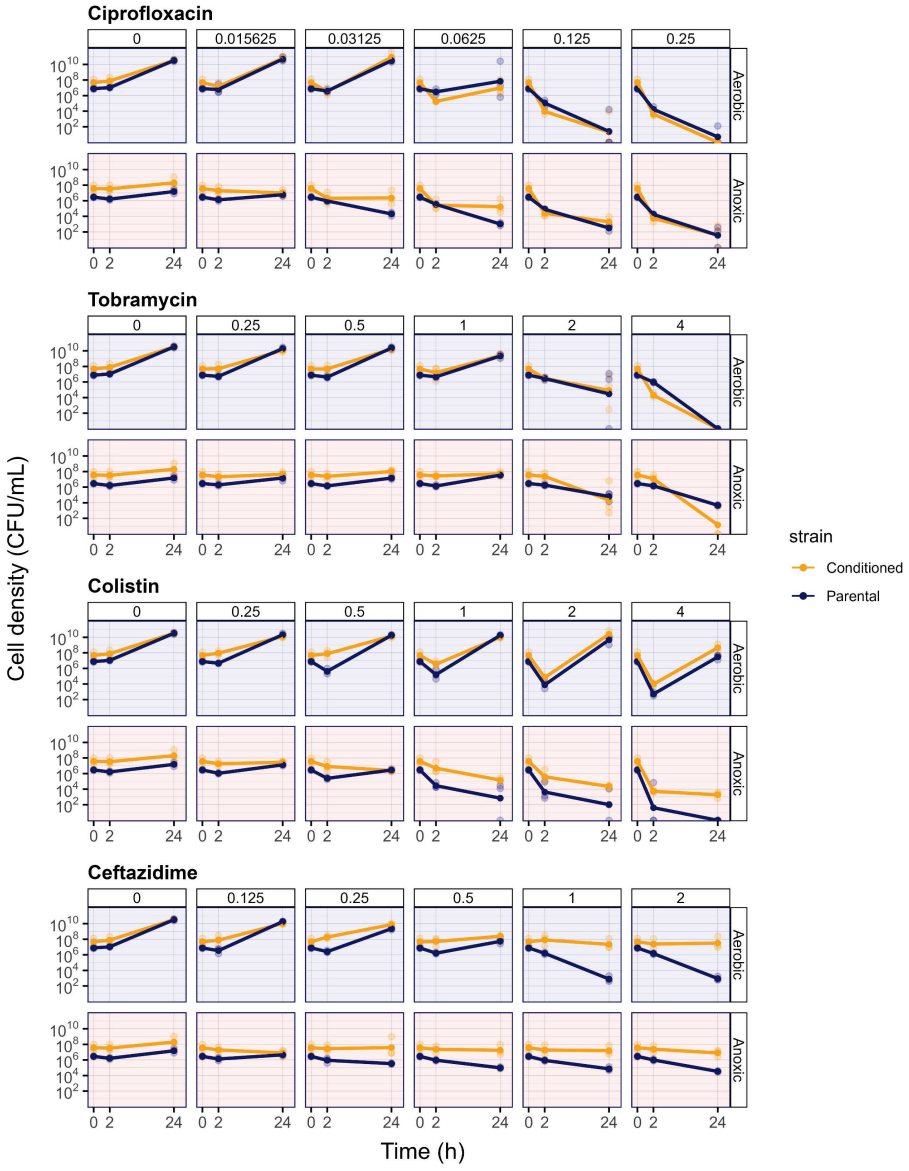
4.6. Supplementary materials

Supplemental table 1. Detailed content list of synthetic cystic fibrosis sputum media

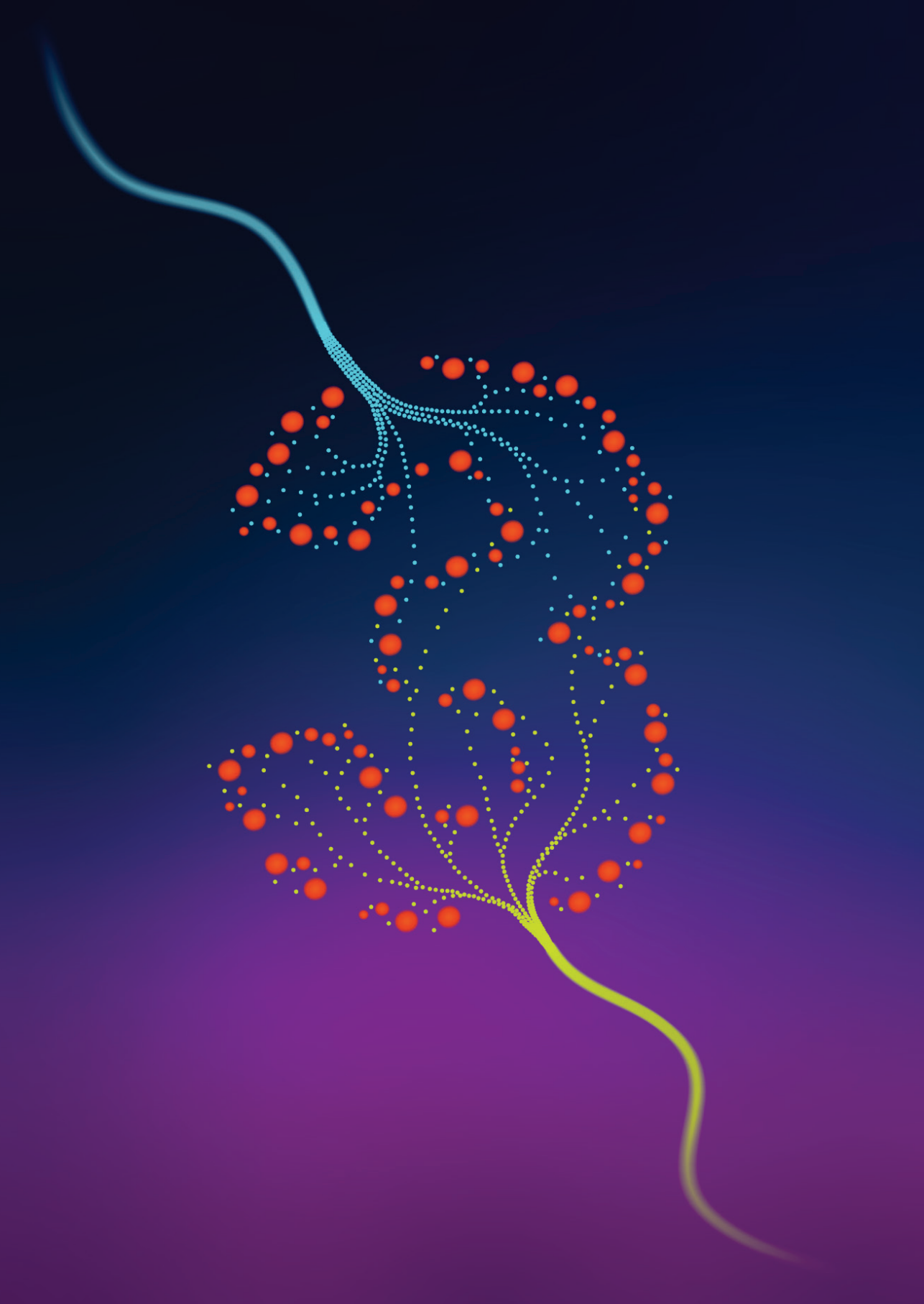
	Name	Concentration (mM)	Company information
M9 buffer	di-sodium hydrogen phosphate (Na_2HPO_4)	90.2	Thermo Fisher Scientific
	Potassium di-hydrogen phosphate (KH_2PO_4)	22.0	VWR International
	Sodium chloride (NaCl)	8.5	Merck KGaA (Avantor™)
	Ammonium chloride (NH_4Cl)	18.6	Alfa Aesar
	Magnesium sulphate hepta-hydrate ($MgSO_4$)	1.0	VWR International
	Calcium chloride ($CaCl_2$)	0.1	Acros Organics
Salts	Potassium nitrate (KNO_3)	0.35	Acros Organics
	Iron sulphate ($FeSO_4$)	0.0036	Alfa Aesar
Suppl.	BME Vitamin solution	*1x*	Thermo Fisher Scientific
Trace metals	Di-sodium Ethylene di-amine tetra-acetic acid (EDTA)	0.002 (mg/mL)	J.T. Baker (Avantor™)
	Zinc Sulphate hepta-hydrate ($ZnSO_4$)	0.23 (mg/mL)	Alfa Aesar
	Boric acid (H_3BO_3)	0.111 (mg/mL)	Acros Organics
	Manganese chloride tetra-hydrate ($MnCl_2$)	0.051 (mg/mL)	Sigma Aldrich (Avantor™)
	Cobalt chloride ($CoCl_2$)	0.017 (mg/mL)	Alfa Aesar
	Copper Sulphate penta-hydrate ($CuSO_4$)	0.015 (mg/mL)	Sigma Aldrich (Avantor™)
	Ammonium hepta-molybdate tetra hydrate ($(NH_4)_6\ Mo_7O_2$)	0.01 (mg/mL)	Alfa Aesar
Nutrients	Alanine (Ala)	1.8	Chem-Impex International
	Arginine (Arg)	0.3	Chem-Impex International
	Aspartate (Asp)	0.8	Chem-Impex International
	Cysteine (Cys)	0.2	Chem-Impex International
	Glucose (GLC)	3.2	Alfa Aeser
	Glutamate (Glu)	1.5	Chem-Impex International
	Glycine (Gly)	1.2	Acros Organics
	Histidine hydrochloride (His)	0.5	Chem-Impex International
	Isoleucine (Ile)	1.1	Chem-Impex International
	Lactate (LAC)	9.0	Biosynth International
	Leucine (Leu)	1.6	Chem-Impex International
	Lysine hydrochloride (Lys)	2.1	Thermo Fisher Scientific
	Methionine (Met)	0.6	Chem-Impex International
	Phenylalanine (Phe)	0.5	Chem-Impex International
	Proline (Pro)	1.7	Thermo Fisher Scientific
	Serine (Ser)	1.4	Chem-Impex International
	Threonine (Thr)	1.0	Chem-Impex International
	Tryptophan (Trp)	0.01	Chem-Impex International
	Tyrosine (Tyr)	0.8	Chem-Impex Internationa
	Valine (Val)	1.1	Chem-Impex International

Supplemental table 2. Median minimal inhibitory concentrations (MIC) of antibiotics for the parental *P. aeruginosa* strain (n=3 biological replicates). MICs were determined after 20 hours of aerobic incubation at 37 °C with shaking (250 rpm).

Strain	Ceftazidime	Colistin	Ciprofloxacin	Tobramycin
PAO1 (DSM1117)	0.5 mg L^{-1}	4 mg L^{-1}	0.125 mg L^{-1}	1 mg L^{-1}



Supplemental figure 1. Antibiotic time-kill assays comparing parental and anoxic conditioned *P. aeruginosa* under anoxic and aerobic conditions. Mean cell densities over time are shown with solid navy blue points and lines for the parental PAO1 strain, and orange points and lines for the anoxic conditioned strain. The means were calculated from the log-transformed cell densities of three biological replicates, represented by translucent points. Background shading indicates treatment conditions, with blue for aerobic and orange for anoxic environments.



Section III

Nutrients shape antibiotic resistance evolution

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 5

Unraveling antimicrobial resistance using metabolomics

Maik Kok, Loes Maton, Marielle van der Peet,

Thomas Hankemeier, Coen van Hasselt

Drug Discovery Today, 2022, 27(6), 1774-1783

Teaser

Novel treatment strategies are needed to address the emerging threat of antimicrobial resistance (AMR) in bacterial pathogens. Metabolomics approaches may help to unravel biochemical underpinnings of AMR, to facilitate the discovery of metabolism-associated drug targets and treatment strategies.

Abstract

The emergence of antimicrobial resistance (AMR) in bacterial pathogens represents a global health threat. The metabolic state of bacteria is associated with a range of genetic and phenotypic resistance mechanisms. This review provides an overview of the role of metabolic processes associated with AMR mechanisms including energy production, cell wall synthesis, cell-cell communication, and bacterial growth rate. These metabolic processes can be targeted to re-sensitizing resistant pathogens for antibiotic treatments. We discuss how state-of-the-art metabolomics approaches can be used for comprehensive analysis of microbial metabolism concerning AMR, which may facilitate the discovery of novel drug targets and treatment strategies.

5.1. Introduction

Antimicrobial resistance (AMR) in bacterial pathogens represents an urgent global health threat associated with significant morbidity and mortality[1]. To this end, there exists a need to improve our understanding of underlying molecular mechanisms of AMR to develop innovative treatment strategies for AMR-associated bacterial infections[2].

Bacterial pathogens can survive antibiotic exposure through a range of genetic and phenotypic AMR mechanisms. Genetic mechanisms are associated with a permanent change in antimicrobial sensitivity, for example due to the acquisition of mobile genetic elements and mutations in chromosomal genes conferring antibiotic resistance[3]. Phenotypic mechanisms are typically linked to transiently decreased antibiotic sensitivity in either a homogeneous (e.g., tolerance) or heterogeneous fashion(e.g., heteroresistance, persistence)[4–6]. Another phenotypic mechanism which decreases antibiotic sensitivity is the formation of microbial biofilms, which are aggregates of bacteria protected by a polymeric matrix.[7]Importantly, the prolonged antibiotic survival of bacteria through phenotypic AMR mechanisms may act as a stepping-stone for genetic AMR development.[8]Bacterial metabolic processes have a fundamental role in cellular function and are therefore commonly associated with various AMR mechanisms (**Figure 1**). The metabolic state of bacterial cells during antibiotic treatment can either as a contributor to or as a consequence of AMR. Decreased metabolic activity contributes to AMR by reducing antibiotic uptake or secondary effects of antibiotics[9–12]. In contrast, increased metabolic activity is required to support energy-demanding AMR mechanisms such as cell-wall modifications and efflux pumps overexpression[13–18]. Understanding these underlying metabolic processes of AMR mechanisms may be used to strategically alter metabolic activity during antibiotic therapy to re-sensitize pathogens[19]. Metabolomics approaches represent a key enabling technology to help identify relationships between AMR mechanisms and microbial metabolism. Metabolomics represents the systemic study of the metabolome, all small molecules in a biological sample, providing a snapshot of the utilized biochemical processes[20,21]. The metabolome is a close link to organismal phenotype, unveiling initial responses to antibiotic pressure and the adaptations required to sustain AMR mechanisms.

In this review we discuss the role of bacterial metabolism in AMR mechanisms and microbial biofilms for clinically relevant bacterial pathogens, using state-of-the-art metabolomics approaches. Secondly, we discuss how metabolomics may be applied as a key enabling technology to facilitate the discovery of innovative metabolism-associated drug targets and treatment strategies.

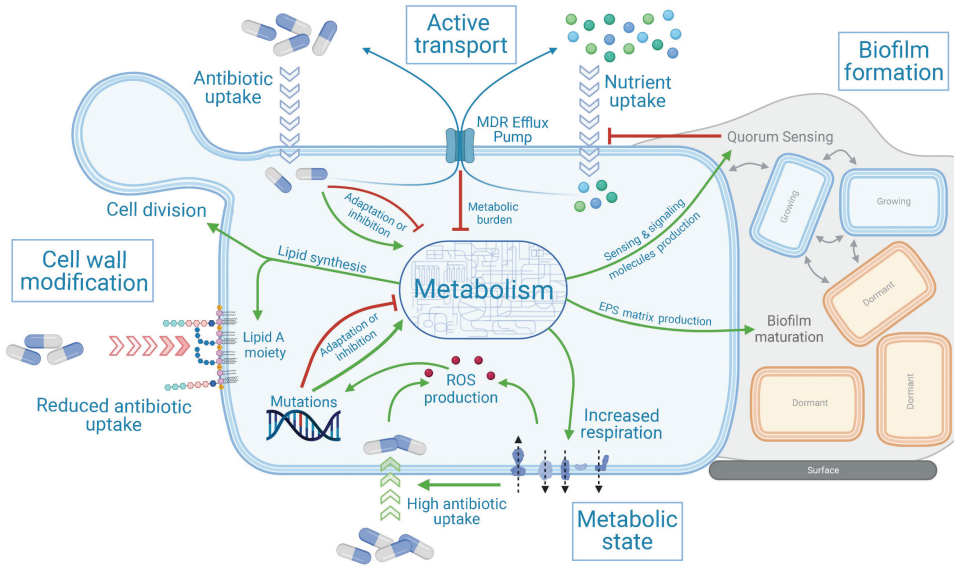


Figure 1. Schematic overview of the role of bacterial metabolism concerning antimicrobial drug action and resistance mechanisms.

5.2. Metabolism and antimicrobial resistance

Key cellular changes associated with AMR where metabolism plays an important role include (i) cellular energy production, (ii) cell envelope modifications, and (iii) cell-to-cell interactions in biofilms. Here, we provide an overview of metabolomics studies that have identified such AMR-associated metabolic effects (**Table 1**).

Table 1. Overview of studies researching the role of metabolism in AMR using metabolomics techniques

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
Energy metabolism influences antibiotic efficacy	Energy production is a better predictor for antibiotic efficacy compared to the growth rate	Nucleotides	Colorimetric*	Ampicillin[+] Carbenicillin[+] Gentamicin[±] Kanamycin[±] Streptomycin[±] Ciprofloxacin[<] Levofloxacin[<] Norfloxacin[<] Cefsulodin[>]	*E. coli*	37
	Bacteriostatic antibiotics inhibit the efficacy of bactericidal antibiotics due to the reduced energy demand of treated cells	Amino acids Nucleotides	Untargeted LC and GC-MS	Ampicillin[+] Gentamicin[±] Levofloxacin[<] Norfloxacin[<] Daptomicin[>] Rifampin[>]	*E. coli* *S. aureus*	30
Nutrient supplementation	Antibiotic resistant cells reduce activity in central carbon metabolism, which can be activated by nutrient supplementations	Glycolysis TCA cycle	Untargeted GC-MS	Kanamycin[±]	*E. tarda*	31,32
	Supplementation of TCA cycle stimulating nutrients increase aminoglycoside tobramycin proton motive force induced cellular intake	TCA cycle	Untargeted LC and GC-MS	Tobramycin[±] Chloramphenicol[>] Linezolid[>] Rafampin[>]	*E. coli*	25
Respiration and secondary antibiotic effects	Resistant cells demonstrate lower levels of TCA cycle intermediates, reducing ROS production	Amino acids Glycolysis Lipids TCA cycle	Untargeted LC-MS	Chloramphenicol[>]	*E. tarda*	42
	Decreased central carbon metabolites in antibiotic resistant cells reduce ROS production	Amino acids Glutathione Glycolysis Pentose phosphate TCA cycle	Untargeted GC-MS	Gentamicin[±]	*V. alginolyticus*	43
	Energy metabolism as a defense mechanism to reduce oxidative stress during antibiotic treatment	Glycolysis TCA cycle	(Un-) targeted LC-MS	Streptomycin[±] Isoniazid[>] Rifampicin[>]	*M. tuberculosis*	45
Biofilm heterogeneity	QS can slow down cell growth by coordinating nucleotide production and glucose utilization	Nucleotides Pentose phosphate	Targeted NMR and CE-MS	N.A.	*B. glumae*	67
	Cells in the biofilm core switch to anaerobic fermentation for energy production	Lactate TCA cycle	Targeted NMR	N.A.	*S. oneidensis*	76

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
Metabolic adaptation in energy production		Nutrient consumption	Targeted LC-UV and LC-MS	N.A.	*P. aeruginosa*	40
	High nutrient levels promote antibiotic resistance development of resistance	Lipids Glycolysis TCA cycle	Untargeted MS	Ampicillin[+] Norfloxacin[<] Chloramphenicol[>]	*E. coli*	38
	Antibiotic treatment from different classes results in distinctive metabolic perturbations and adaptation	Amino acids Glycolysis Nucleotides TCA cycle	Untargeted MS	Kanamycin[±] Nalidixic acid[<] Norfloxacin[<] Ofloxacin[<] Chloramphenicol[>]	*E. coli*	63
	Resistant and sensitive bacteria have distinctive metabolic fingerprints	Glycolysis Lipids Nucleotides Pentose phosphate TCA cycle	Untargeted and targeted LC-MS	Methicillin[+]	*S. aureus*	44
Antibiotic induced metabolic adaptation	Metabolic fingerprints identify antibiotics (secondary) mode of action for different antibiotic classes	Amino acids Nucleotides TCA cycle	Untargeted NMR	Ampicillin[+] Carbenicillin[+] Ciprofloxacin[<] Ofloxacin[<] Streptomycin[±] Cefalexin[>] Doxycycline[>] Tetracycline[>]	*E. coli*	61
	Antibiotics and their corresponding mode of action can be identified based on the targeted metabolic pathways of these antibiotics	Amino acids Glycolysis Lipids Nucleotides TCA cycle	Untargeted LC-MS	Ceftazidime[>] Fosmidomycin[>] Triclosan[>]	*E. coli*	62
	Antibiotic surviving cells actively produce ATP during antibiotic treatment, dependent on the nutritional environment	Nucleotides	Colorimetric*	Ciprofloxacin[<] Streptomycin[±] Bedaquilline[>] Isoniazid[>] Rifampicin[>]	*M. smegmatis*	100
	Methicillin resistant and sensitive strains demonstrate different metabolic responses to treatment with other antibiotics	Amino acids Nucleotides TCA cycle	Targeted LC-MS	Ampicillin[+] Ciprofloxacin[<] Kanamycin[±]	*S. aureus*	81
	Antibiotics induce microbiome-independent changes in the host metabolome which alter antibiotic efficacy	Amino acids Glycolysis Nucleotides Pentose phosphate	Untargeted LC-MS	Ciprofloxacin[<]	*E. coli*	41

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
Biofilm formation	There is a heterogeneous distribution of quorum sensing molecules over the biofilm population	Quorum sensing	Targeted MALDI-SIMS	N.A.	*P. aeruginosa* *S. aureus*	72
	The production of antimicrobials and signaling molecules is influenced by the nutritional environment	Quorum sensing	Targeted MALDI-SIMS	N.A.	*P. aeruginosa* *S. aureus*	79
Cell envelope modifications						
Cell wall disruption and synthesis	The loss of envelope and membrane biogenesis processes results in complete lipid reconstruction, including changes in lipid A moiety, resulting in the energy metabolic switch to glycolysis	Amino acids Lipids Pentose phosphate TCA cycle	Untargeted (LC-)MS	Colistin$^{\Delta}$	*K. pneumoniae*	55
	Lipid A reconstruction results to increase pentose phosphate activity and reduced TCA cycle activity in colistin resistant cells	Lipids Pentose phosphate TCA cycle	Untargeted LC-MS	Colistin$^{\Delta}$	*A. baumannii*	56
	Colistin treatment induces metabolic flux towards cell wall repair, forcing the energy production flux to glucose utilization and shuttled TCA cycle	Glycolysis Lipids TCA cycle	Untargeted GC-MS	Colistin$^{\Delta}$	*M. tuberculosis*	57
	Combination therapy with colistin and doripenem antibiotics affect metabolic pathways in cell wall synthesis and energy production differently in a time-dependent manner	Amino acids Glutathione Lipids Nucleotides Pentose phosphate	Untargeted LC-MS	Colistin$^{\Delta}$ Doripenem^{+}	*A. baumannii*	64
	The addition of phosphoethanolamine to lipid A for colistin resistance has a high fitness cost	Lipids	Targeted MALDI-MS	Colistin$^{\Delta}$	*E. coli*	59
MDR overexpression	The overexpression of MDR efflux pumps initiates metabolic rewiring to anaerobic respiration	Oxygen and nitrates	Colorimetric* Oximeter	N.A.	*P. aeruginosa*	17

*Fluorescent staining of targeted metabolites

$^{+}$β-Lactam antibiotics

$^{<}$Fluoroquinolone antibiotics

$^{\pm}$Aminoglycoside antibiotics

$^{\Delta}$Polymixin antibiotics

$^{>}$Other antibiotic classes

Cellular energy production

The activity of energy producing metabolic pathways translate the activation of cellular functional responses or dormancy to evade antibiotic killing. The most energy efficient producing metabolic pathway is aerobic cellular respiration[22].

Cellular respiration includes glycolysis and the tricarboxylic acid (TCA) cycle for the production of electron carriers, which are used in the electron transport chain (ETC) for the production of adenosine triphosphate (ATP). In case of fast energy demand or carbon source depletion, several bacterial pathogens may switch towards less efficient anaerobic fermentative energy production[22]. Several pathogens such as *Pseudomonas aeruginosa* can utilize anaerobic metabolic respiration such as the nitrate respiratory chain to maintain cellular homeostasis in oxygen-depleted environments.[23] A switch towards anaerobic energy metabolism is commonly used for evasion of host defense mechanisms but also plays an important role to increase aminoglycoside tolerance[24,25]. For example, increasing oxygen levels using hyperbaric oxygen treatment (HBOT) to induce aerobic respiration re-sensitizes *P. aeruginosa* to aminoglycoside treatment[26,27]. However, this approach is only of interest in specific clinical indications, e.g. anaerobic microenvironments in cystic fibrosis-associated lung infections.

Stimulation of aerobic energy production as a therapeutic target to enhance antibiotic sensitivity is an important potential therapeutic strategy. Specifically, supplementation of essential carbon sources to increase aerobic respiration[19], is a promising novel approach to improve antibiotic efficacy, in particular for aminoglycoside antibiotics. Comprehensive *in vitro* screens in bacteria using different carbon source supplements have demonstrated pathogen-dependent changes in aminoglycoside susceptibility with nutrient supplementation[28]. Metabolomics studies demonstrated such carbon source supplementation changes the TCA cycle activity for the synthesis of electron carriers to support the ETC[29–32]. Aminoglycoside efficacy can be increased by increasing the passive influx of charged molecules. Stimulating the ETC results in a higher electric transmembrane potential which enhances the proton-motive force (PMF) mediated influx of the positively charged aminoglycosides[29,33]. Increasing the antibiotic uptake by nutrient-induced PMF demonstrated decreased cell survival in several multi-drug resistant strains.[31]Other antibiotic classes, like β-lactams and fluoroquinolones, also partly depend on cellular respiration for their antimicrobial effects by inducing a redox disbalance as a secondary antibiotic effect. Fluoroquinolones exert better bactericidal effects in metabolic active cells by the production of reactive oxygen species (ROS) during oxidative phosphorylation[11,12,34]. β-Lactams induce systemic ROS-associated cellular toxicity by creating an energy-demanding futile cycle of peptidoglycan

synthesis and degradation by obstructing cell wall synthesis[35,36]. The close kinship between energy production and antibiotic lethality is further demonstrated by the increased bactericidal killing in cells with accelerated respiratory activity[30]. In accordance, metabolic activity has experimentally shown to better forecast antibiotic effect than growth rate[37].

It needs to be taken into account that nutrient supplementation to increase antibiotic uptake and induce secondary antibiotic effects rely on the metabolic specialization of the targeted bacterial cell. Clinically relevant strains potentially lose the ability to utilize certain pathways during the acquisition of antibiotic resistant conferring mutations and adaptation to specific microenvironments at infection sites[38–41]. Antibiotic resistant strains demonstrate distinctive metabolic footprints[42–44]. The observed decline in energy metabolism reduces ROS production thereby further enhancing AMR[45]. Subsequently, limiting mutations in core metabolic genes directly results in the development of antibiotic resistance[46]. However, metabolism affecting mutations, such as PMF-limiting mutations, can only be sustained in nutrient-rich environments due to the high fitness burden[47]. To further unravel such metabolic effects and adaptations associated with antibiotic efficacy, the use of mathematical flux analysis of central metabolic pathways could help to scrutinize the effect of nutritional supplements on metabolic processes during antibiotic treatment[31,48]. Although these approaches targeting cellular energy metabolism are of interest, there remains a significant knowledge gap concerning the broad spectrum of bacterial species and clinically occurring strains.

Cell envelope modifications

Cell wall permeability is essential for effective antibiotic treatment since most antibiotics rely on passive transport across the outer membrane[49]. In particular for Gram-negative pathogens, the cell wall can be challenging to penetrate by antibiotics, in part due to the outer layer of negatively charged lipopolysaccharides (LPS), preventing passive transport over the cell wall for large and hydrophobic antibiotics. The uptake of antibiotics in gram-negative pathogens to exert their effect is mainly dependent on transport through membrane porins. Here, porin permeability is higher for positively charged small molecule antibiotics, possibly because of the role of the discussed PMF[50].

The LPS layer in gram-negative pathogens is moreover an important drug target to disrupt cell envelope integrity, where modifications in LPS lead to AMR. Polymyxin antibiotics, currently used as last resort antibiotics, bind lipid A in the LPS layer of gram-negative bacteria to initiate lethal disruption of both outer and cytoplasmic membranes, and increase intracellular levels of combination therapeutics[51,52]. Modifications to lipid A structure through changes in the biosynthetic pathway of LPS can lead to resistance to polymyxins. Polymyxin resistance mechanisms include active membrane modifications to reduce the lipid A binding sites in the LPS layer, through intrinsic adaptation, acquired chromosomally encoded, and plasmid-mediated[53–55]. The process of cell modifications is supported by a wide range of fatty acid biosynthetic pathways[13]. Rewiring of fatty acid synthesis, however, comes with a high energy demand, which is demonstrated by the increased killing efficiency in metabolic inactive cells[56]. Metabolomics studies of polymyxin resistant strains demonstrated that modifications in lipid biosynthesis result in metabolic rewiring in energy metabolism[57,58]. Metabolic flux analysis in another strain supports this finding, as the upper carbon flux in the glycolysis pathways was elevated while the TCA cycle was shunted[59]. This suggests the switch to glucose-fermenting metabolism for energy production polymyxin resistant cells, supported by the use of pH-mediated detection of lactic acid producing polymyxin resistant *Enterobacteriaceae*[60]. Although fermentative metabolism can sustain cell homeostasis, the high metabolic burden of fatty acid synthesis during resistance acquisition results in a fitness cost[14,15,61]. To this end, enhancing our understanding of the biosynthetic routes of LPS and fitness cost during polymyxin resistance can potentially improve the development of drug candidates targeting the cell envelope.

The overexpression of multidrug resistance (MDR) efflux pumps in the cell envelope is another mechanism to regulate intracellular concentrations of antibiotics leading to AMR and a fitness cost. MDR efflux pump-associated AMR occurs for a range of broad antibiotic classes across pathogenic species[62]. Metabolic rewiring is an important enabling mechanism to overcome metabolic burden accompanied by MDR efflux pump overexpression[16–18]. For instance, the switch towards the nitrate respiratory chain and anaerobic fermentative metabolism compensates for the use of oxygen as an alkaline agent, which enables the acquisition of MDR efflux pump promoting mutations in the absence

of selective pressure.[17] The reliance on metabolic adaptation to maintain cellular homeostasis during AMR mechanisms could potentially be utilized therapeutically. For instance, metabolic adaptation upon antibiotic exposure[63–65] can be used to design combination treatments with antibiotic agents. Antibiotics affect different key metabolic pathways, disrupting cell homeostasis, expected to be one of the driving forces between the synergistic effect of the combination therapy of colistin and doripenem[66]. In conclusion, targeting metabolic changes due to efflux pump upregulation are of interest to target therapeutically.

Cell-cell interactions in biofilms

The formation of microbial biofilms forms an important mechanism to decrease antibiotic sensitivity, through the production of extracellular polymeric substances (EPS). Production of EPS is however a metabolically expensive activity, which requires efficient cellular communication and metabolic adaptation[67]. Bacteria utilize quorum sensing (QS) systems to coordinate cell-cell interactions in all biofilm stages. QS occurs through the production of various hormone-like small molecules excreted in the biofilm microenvironment and is essential in biofilm formation and maintenance by synchronizing metabolism for the production of macromolecules to establish the protective layer of extracellular polymeric substances (EPS) layer[68,69]. Targeting QS-associated metabolic processing may thus represent an important target for biofilm-associated infections.

A promising approach to improve the treatment of biofilms exploits the role of QS molecules in biofilm physiology. Disruptive microbial communication treatments can interfere with biofilm integrity over multiple biofilm stages[70], enabling treatment approaches for different stages of infection. The link between metabolic activity in biofilms and QS is can also be utilized to disrupt biofilm integrity. The use of QS-controlled circuits for dynamic control of cellular fluxes[71] demonstrates that cell-to-cell communication is a key regulator of bacterial metabolism, which indirectly affects antibiotic susceptibility. Therefore, QS systems create an opportunity to be used as a treatment target[72,73] to get a universal control over metabolic-associated antibiotic potentiation and biofilm physiology. However, the high variety of QS systems and differences between species require further identification and characterization of QS molecules which partake in biofilm biology. Spatial-oriented mass spectrometry techniques can

identify utilized QS molecules and characterize population dynamics in biofilms[74].

Chronic bacterial infections are commonly associated with well-developed mature biofilms and are associated with reduced antibiotic efficacy. In particular well-developed mature biofilms are associated with steep nutrient gradients induced by the biofilm structure[75]. The biofilm maturation process is often dependent on the ability of pathogens to metabolically switch to alternative nutrient sources[76,77]. Real-time analysis of the metabolites from the central carbon metabolism demonstrated metabolic adaptations to anaerobic fermentation pathways over time and biofilm depth[78]. Redirecting metabolism in *P. aeruginosa* biofilms by TCA cycle carbon source supplementation has resulted in increased aminoglycoside eradication[29,33], which highlights the potential of nutrient supplementation to reduce metabolic induced tolerance in biofilms[7].

5.3. Metabolomics technologies and approaches

Metabolomics approaches enable organism-wide metabolite identification and quantification of biochemical networks. Metabolomics approaches can be broadly differentiated into untargeted metabolite profiling and targeted methods. Untargeted methods aim for broad metabolite coverage, but may not allow full identification of molecular structures. Targeted metabolomics approaches aim for quantitative analysis for a set of metabolites, with enhanced possibilities for structural resolution of identified metabolites.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most commonly used detectors in the metabolomics field. MS systems generally have superior selectivity, sensitivity, and can detect a larger range of analytes. The detection is based on the manipulation of ionized analytes by an electric or magnetic field to obtain the mass-to-charge (m/z) ratio. The charge-dependent detection requires the ionization of metabolites in the ion source before entering the MS system. NMR detectors provide, complementary to MS, quantitative and structural information in a non-destructive manner.

Even though metabolomics technologies advanced extensively over the years, the analysis of the full organismal metabolome in a single analytical method is still not possible due to the high diversity in physicochemical characteristics and broad range of concentrations of the metabolites. Here, we

discuss practical considerations considering the utility of different metabolomics approaches in particular in the context of microbial metabolism and AMR (**Table 1**).

Untargeted metabolomics

Untargeted non-selective screening enables the broad characterization of (changes in) metabolism. The analysis of multiple metabolite classes, with high contrast in polarity, requires long separation methods to maintain accurate metabolite identification. The use of high-resolution mass spectrometers (HRMS) enables high throughput metabolite profiling without the need for combining multiple or time-consuming analytical platforms. HRMS refers to mass analyzers, such as time-of-flight, Orbitrap, and Fourier-transform ion cyclotron resonance, with high mass accuracy, dissociating metabolites up to 0.001 atomic mass units. This high metabolite resolving power of HRMS facilitates the confident identification of metabolites to study metabolic changes without a time-consuming separation step and confident comparison of acquired m/z features to the masses of previously identified metabolites stored in mass spectral libraries[79]. This enables relatively fast metabolic fingerprinting, which can be used to screen for metabolic adaptation during AMR development in a larger set of conditions with higher throughput. The high throughput provided the possibility to research metabolic evolution and immediate metabolic response during antibiotic treatment with a variety of antibiotic classes within single studies[38,65]. Comparison studies between bacterial species, environments, or antibiotic classes relying on metabolic data measured within a single do not fully rely on data acquired in other studies, reducing the variability caused by differences in experimental design.

The high mass accuracy achieved with HRMS can also be used to assign the molecular composition of completely unknown metabolites, which was for instance applied for the discovery of novel metabolites. All metabolites covered in central carbon metabolism are covered in most metabolite databases, while many secondary metabolites like QS molecules are yet to be discovered[80]. However, the robust identification of chemical structures requires the addition of low-resolution fragmenting mass analyzers or the multidimensional information from NMR detectors. This metabolite identification method combined with spatial oriented ionization techniques demonstrated the influence of the

nutritional environment and biofilm formation in the production of signaling molecules[74,81]. Thus, the advances in HRMS technologies provide the opportunity to confidently screen for metabolic adaptations or unidentified metabolites concerning AMR, which can be used to better understand AMR mechanisms or develop metabolism-targeted treatment strategies[82].

Targeted metabolomics

Targeted metabolomics approaches require prior knowledge of metabolite targets to enable efficient extraction and isolation from the sampled cells, providing sensitive and selective quantitative analytical methods. This is in contrast with untargeted methods which come with the bottleneck of limited detection range and quantitative accuracy due to detector saturation by thousands of signal-producing analytes. The confident identification and high sensitivity of targeted metabolomics enables the characterization of exact changes in metabolites concentrations. However, this requires the use of expensive or complex standardization procedures and time consuming analytical validation. The specialization of targeted methods limits the metabolic targets, which results in studies focusing on specific metabolic pathways[40,45,69,78,83]. Nevertheless, the absolute quantitative data obtained in targeted metabolomics are superior for biological interpretation. For example, the quantitative analysis of nutrient uptake and metabolism with both NMR and MS was combined with earlier obtained RNA sequencing data to determine QS-controlled metabolic repression[69]. This study was not able to analyze broad spectrum of potential carbon or nitrogen sources in the nutrient-rich culture medium, impeding the full characterization of the metabolic phenotype, which can be addressed by using a combination of analytical methods or elaborate targeted methods using chemical derivatization[84,85]. Targeted metabolomics platforms enable the interpretation of metabolite utilization during or after AMR development, in particular for metabolic flux studies using isotope labeling. The isotope labels in core nutrients can be followed over time until a metabolic steady state is achieved, providing information about enzyme function and metabolite transport through various metabolic pathways[86]. The high precision of targeted methods is of utmost importance as changes in measured metabolite levels influence the metabolic network model. The metabolic networks in combination with transcript and protein changes are key for the understanding of cellular

regulatory systems. For example, extensive fluxomics research demonstrated different metabolic specialization on physiological relevant carbon sources during infection in the cystic fibrosis lung[24]. Similar metabolic flux adaptation was observed during AMR development, which was successfully targeted using nutrient supplementation[31]. The targeted analytical methods used in metabolic flux studies benefit from the high sensitivity and metabolite coverage of MS detectors hyphenated to separation methods but can be transferred to NMR to determine nutrient exchange and utilization on an intercellular level. The non-destructive nature of NMR detectors enables the real-time quantification of metabolic fluxes in living samples, used for studies on nutrient interchange between biofilm sub-populations[78].

Sample preparation

Metabolomic data should represent the metabolic state of the microbial population at the moment of sample collection. Metabolic quenching is a critical initial step in the sample preparation process to provide an unbiased snapshot of metabolism, given that many metabolites have a rapid turnover rate[87]. Especially the role of energy metabolism in AMR mechanisms demands efficient quenching techniques as energy and electron carrying molecules are chemically labile metabolites with extremely high turnover rates. Quenching methods need to be chosen based on the cell-wall composition of the strain of interest to prevent the leakage of intracellular metabolites[87,88].

Composing the further sample preparation steps consists of the choice for the metabolite extraction procedure and the sample clean-up method. The extraction of intracellular metabolites can be achieved by the chemical or mechanical lysis of the cell wall[87,89,90]. Chemical lysis reduces the metabolite degradation or leakage of macromolecules but needs to be chosen based on the analytes of interest. Here, the polarity of the lysis solvent influences the extraction efficiency of different metabolite classes. For example, a study on the influence of colistin treatment on membrane profiles and energy metabolites uses two different extraction methods[57]. Combining chemical lysis with mechanical cell disruption is another method to increase the metabolite coverage of the analytical method. Changes in the sample extraction method and targeted bacterial species can impact the extraction efficiency differently per metabolite[90]. The final step of the sample preparation procedure is sample clean-up, in

particular important for MS-based methods. The ionization step in MS can be interfered by common components such as salts, sugars, lipids, and proteins[91]. The ionization suppressing elements can be removed by non-selective protein precipitation or optimized techniques such as liquid-liquid extraction and solid-phase extraction[91,92]. The sample preparation decisions are dependent on the analytical approach since untargeted metabolomics approaches aim for high metabolite coverage achieved with non-selective sample preparation, and targeted metabolomics aims for sample preparation methods resulting in high recovery values for the analytes of interest[93]. Importantly, the development and use of standardized protocols in sample preparation techniques are beneficial for the comparison of metabolomics data between studies because of the dynamic nature of metabolism.

Future perspectives on metabolomics technologies

Because metabolomics is closely related to biological phenotype it is therefore expected to be essential tool to unravel the phenotypical AMR mechanisms and metabolic adaptations during genetic AMR. The integration of metabolomics with microfluidic systems, enables further elucidation of the complex communication systems[94,95]. Metabolomic analysis of co-cultivated strains and their environment can be used to study small molecule virulence factors, such as QS, in both commensal and competitive interactions, and their effect on metabolic diversity during host colonization. Here, the advances in resolution and sensitivity of MS analysis can enable both the identification of QS molecules and the elucidation of the metabolic footprint.

The next fundamental step in unraveling phenotypic heterogeneity and their role in AMR mechanisms is the characterization of metabolic profiles from single cells within a heterogeneous population[96]. Slow growth and dormancy are considered essential in antibiotic tolerant or persistent subpopulations[5,97,98]. However, the metabolic activity in these cells and its role in AMR is still debated[7,97,99,100]. NMR imaging and spatial ionization techniques demonstrated different metabolite profiles within bacterial populations, but currently lack the resolution to scrutinize the contribution of single bacterial cells. A prevalent single cell technique attains its resolution by sampling one cell in an ionization probe before MS analysis, where metabolic coverage is mainly dependent on MS resolution or the integration of innovative separation techniques such as ion

mobility[101]. Applying these techniques in resolving bacterial heterogeneity during antibiotic treatment requires multiple improvements to handle low bacterial intracellular volumes and the stochastic distribution.

5.4. Conclusion

Metabolic changes due to evolution and phenotypic adaptation at the infection site are associated with a broad range of AMR mechanisms. Enabling metabolomics technologies can help further unravel and characterize these AMR-associated metabolic effects. So far, metabolomics studies have however focused on a limited number of bacterial species and antibiotics. Systematic application of metabolomics studies in conjunction with complementary next-generation sequencing approaches and experimental evolution models in clinically relevant conditions will allow to further unravel the role of microbial metabolism in AMR. The improved understanding may support the discovery of novel metabolism-targeted treatment strategies to be used in combination with established antibiotic agents.

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How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 6

Nutrients drive the antibiotic-specific evolution of resistance in *Pseudomonas aeruginosa*

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Abstract

The pathogen *Pseudomonas aeruginosa* can exploit its metabolic flexibility during cystic fibrosis lung infections to reduce antibiotic sensitivity and offset antibiotic resistance costs, two key traits influencing its evolutionary trajectory. Although each trait has been associated with nutrient conditions, the link between nutrient conditions and antibiotic evolution remains poorly characterized. We examined how single-nutrient conditions influence antibiotic resistance evolution in *P. aeruginosa* through phenotypic and genotypic adaptations. We used adaptive laboratory evolution with different antibiotic classes in single nutrient media, we then compared these results to those obtained in nutrient-rich synthetic cystic fibrosis sputum medium (SCFM). Antibiotic susceptibility testing after evolution showed limited differences in minimal inhibitory concentrations (MIC) between single nutrient conditions for ceftazidime and imipenem, but more pronounced impact for ciprofloxacin, colistin, and tobramycin. Ciprofloxacin evolution resulted in the highest MIC increase, with at least a 4-fold increase observed in glutamate-evolved lineages, whereas glucose-evolved lineages showed up to 4-fold reduction in MICs for tobramycin, compared to lineages evolved under all other nutrient conditions for the same antibiotic. Growth kinetics of the evolved strains showed reduced growth rates specific to the antibiotic but not the nutrient condition in itself. Whole-genome sequencing showed nutrient-specific mutational profiles for tobramycin and ciprofloxacin. Tobramycin evolution resulted in glucose specific mutation in *wbpL* and a SCFM-specific mutation in *rplA,* alongside *fusA* and *pmrB* mutations in multiple conditions. Ciprofloxacin resistance was not caused by a *nfxB* mutation in glucose and arginine evolved lineages, which was present in all other lineages, with a specific mutation in *yicC* in the glutamate evolved lineages. No distinct differences between nutrient conditions for colistin were observed. Overall, these findings underscore the significant role nutrient conditions play in shaping resistance and highlight the importance of considering physiologically relevant media when studying antibiotic resistance evolution.

6.1. Introduction

Pseudomonas aeruginosa is the most prevalent pathogen causing chronic infections in the distinctive lung environment of adult cystic fibrosis (CF) patients[1]. This dominance is attributed to the exceptional metabolic versatility of *P. aeruginosa* and its rapid capacity to develop antimicrobial resistance[2–5]. Growing evidence highlights a strong interconnection between these traits, as metabolism influences antibiotic sensitivity and antibiotic resistance mechanisms impose a metabolic burden[6–9]. To advance our understanding of antibiotic resistance evolution, it is crucial to study how nutrients in the environment shape this relationship, a process that remains poorly explored.

In the CF lung, *P. aeruginosa* can utilize a diverse array of nutrients, including amino acids and short-chain fatty acids, whose composition varies substantially among different microenvironments[10,11]. *P. aeruginosa* adapts its metabolism to these varying conditions, which results in class-specific effects on antibiotic sensitivity[12–15]. Altering a single nutrient in the culture condition has been shown to significantly impact antibiotic sensitivity[16]. Because antibiotic sensitivity is a key determinant of selection strength during antibiotic resistance evolution[17], nutrient-induced changes in antibiotic sensitivity can drive the emergence of diverse antibiotic-resistant lineages.

Nutrients also play a role in reducing the fitness cost of antibiotic resistance mutations that alter vital cellular functions, by supporting efficient metabolic rearrangement[18–20]. Consequently, resistance mechanisms with high metabolic burden are less likely to prevail in nutrient-poor environments as this imposes a metabolic constraint[21].

Overall, these studies demonstrate that nutrient environments profoundly influence metabolic adaptations, antibiotic sensitivity, and fitness compensation, all of which shape the evolution of antibiotic resistance. However, the high variability within the CF lung environment, combined with pronounced phenotypic variability, complicates direct comparisons between laboratory conditions and clinical scenarios. Prior research has mainly addressed comparisons between nutrient-rich and nutrient-poor environments concerning fitness landscapes[21,22]. The subsequent essential step is understanding how specific nutrients individually impact resistance evolution. Given the well-documented metabolic flexibility of *P. aeruginosa*[23], accurately evaluating the

influence of individual nutrients within complex media remains challenging. Instead, examining central carbon metabolism responses to single-nutrient conditions can reveal precisely how nutrient-specific adaptations cascade into broader metabolic and biochemical changes[24]. Elucidating how these specific nutrient induced changes influence antibiotic resistance evolution could be essential for optimization or selection of more clinically relevant laboratory conditions.

In this study, we challenged the *P. aeruginosa* metabolic flexibility in single nutrient conditions during antibiotic resistance evolution and explored the phenotypic and genotypic adaptation capabilities in these environments. We conducted adaptive laboratory evolution (ALE) for a range of common antibiotics used to treat *P. aeruginosa* (ceftazidime, ciprofloxacin, colistin, imipenem, and tobramycin), utilizing single nutrient media. The selection of the nutrients arginine, glutamate, glucose, and lactate was based on their physiological relevance in CF mucus and their distinct roles in *P. aeruginosa* metabolism[10,24]. These nutrients have also been shown to affect antibiotic sensitivity differently[16]. Nutrient concentrations were set at 30 mM to prevent nutrient starvation, thereby maintaining stable growth conditions throughout evolution experiments. We assessed phenotypic changes of the nutrient-antibiotic combinations through antibiotic susceptibility testing and growth rate analysis, and genomic changes through whole genome sequencing. To contextualize these findings, we compared the results from single-nutrient conditions to those from a nutrient-rich synthetic CF medium (SCFM), providing insights into how *P. aeruginosa* adapts and maintains metabolic flexibility in minimal environments.

6.2. Material & Methods

Strains and culture conditions

Synthetic CF sputum medium (SCFM) was prepared consisting of physiologically relevant concentrations of nutrients in synthetic CF sputum as described previously[10], 0.11M phosphate buffer, ammonium chloride, potassium nitrate, ferrous sulfate, Basal Medium Eagle 1x vitamins, and trace metals (**Table S1**). Single nutrient media were prepared with the salts, vitamins and trace metals as basal medium, spiked with 4 unique nutrients including arginine, glucose,

glutamate, or lactate, at a concentration of 30 mM. The *P. aeruginosa* PAO1 laboratory strain (DSM 1117; DSMZ, Leibniz Institute, Germany) was used as the parental strain for all evolution experiments.

Antibiotics

Antibiotic stock solutions were freshly prepared on the day of the experiment and diluted to desired concentrations using an Opentrons OT-2 (Opentrons Inc., New York, NY, USA) liquid handling system. Ceftazidime pentahydrate was purchased from Thermo Fisher Scientific (Breda, The Netherlands). Ciprofloxacin, imipenem monohydrate, and tobramycin were purchased from Chem-Impex International (Wood Dale, IL, USA). Colistin sulfate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Laboratory evolution experiment

Three biological replicates of the *P. aeruginosa* starting cell line per media condition were propagated for 10 days under antibiotic pressure to examine the antibiotic resistance development between different culture conditions.

P. aeruginosa PAO1 was streaked out on LB agar plates, and 10 randomly selected colonies were transferred to SCFM (2 mL) and cultured overnight. The liquid cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 to reach the starting inoculation solution, corresponding to an approximate bacterial density of $5*10^6$ CFU/mL. The bacterial inoculum (100 µL) was added to 7 wells with fresh medium with increasing antibiotic concentrations (900 µL) in a 48-well microtiter plate. After 48 hours of incubation, plates were transferred to a BMG microplate reader (Ortenberg, Germany) for OD_{600} acquisition. Cultures at the highest antibiotic concentration reaching the culture density threshold of an OD_{600} of 0.5 were transferred (100 µL) to a new range of antibiotic concentrations (900 µL) in a new 48-well microtiter plate. If the carrying capacity of any of the cultures did not exceed the OD_{600} threshold, the culture under the highest antibiotic pressure reaching at least 80% of the OD_{600} of the positive control was transferred. Cultures under ceftazidime pressure were extensively mixed and the threshold was increased to an OD_{600} of 0.65 due to the build-up of debris in the microtiter plate. At the end of the evolution experiment, the cultures reaching the OD_{600} threshold were transferred (100 µL) to an antibiotic free LB agar plate.

Colonies were collected with a pre-wet swab and stored in 20% glycerol in LB at -80 °C.

Antimicrobial susceptibility testing

Ceftazidime, ciprofloxacin, imipenem, and tobramycin minimal inhibitory concentrations were determined for the parenteral *P. aeruginosa* PAO1 strain, the antibiotic-free lineages, and the lineages evolved under the pressure of the antibiotic in SCFM by a broth microdilution method. Prior to each susceptibility test, fresh subcultures were prepared from the -80 °C stored colonies in 2 mL fresh SCFM. Susceptibility testing was conducted after 24 hours incubation at 37 °C with orbital shaking at 150 rpm or 72 hours including two 20 µL passages into 2 mL fresh SCFM medium. The starting cell density in each condition was approximately 10^6 CFU/mL in a serial twofold dilution of the antibiotics in 96-well microtiter plates (Greiner Bio-one, transparent, flat bottom) with a total volume of 200 µL. The minimal inhibitory concentration (MIC) was defined as the first concentration of antibiotic with no visible growth after 24 hours of incubation at 37 °C.

Growth rate analysis

Growth rate analysis was conducted by culturing the parental *P. aeruginosa* PAO1 strain and all lineages in the single nutrient media and SCFM. The cells were subcultured in 2 mL SCFM and incubated for 24 hours at 37 °C shaking at 150 rpm before dilution to an optical density at 600 nm (OD600) of 0.05 before inoculation. The starting cell density was approximately 10^6 CFU/mL in a transparent 96-well microtiter plate. After inoculation, microtiter plates were transferred to a Liconic StoreX STX44 119 incubator (Mauren, Principality of Liechtenstein) for incubation (95% relative humidity). A Peak Analysis and Automation KX-2 Laboratory Robot (Hampshire, United Kingdom) transferred the microtiter plate every hour between the incubator and the BMG Labtech Fluostar Omega microplate reader (Ortenberg, Germany) for time-course OD_{600} acquisition.

Genome sequencing and bioinformatics

Cells were subcultured in 2 mL SCFM and incubated for 24 hours at 37 °C shaking at 150 rpm. 500 µL of the subculture was pelleted by centrifugation for 10 minutes at 5000 x g. Genomic DNA was extracted using the QIAcube Connect automated

sample preparation system and DNeasy Blood & Tissue Kit extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Extracted DNA was sent to SeqCoast Genomics for short read whole genome sequencing and bioinformatic analysis. Bioinformatic analysis was executed using Breseq (version 0.37.0) for mutation prediction and analysis, Trimmomatic (version 0.39) for read trimming and quality control, and CNOGpro (version deprecated/restored) for copy number variation analysis towards the *P. aeruginosa* PAO1 reference genome. All sequences are stored in the Sequence Read Archive (SRA) database under the PRJNA1217434 identifier[25].

Data Analysis

All data analyses were performed using R. Minimal inhibitory concentrations (MICs) for each evolutionary lineage were determined by calculating the median value from antibiotic susceptibility tests performed in quadruplicate (n = 4 biological replicates). Fold changes in MIC were calculated relative to the median MIC of the parental strain. Antibiotic-free evolution did not result in any significant change in MIC compared to the parental strain (data not shown). To assess differences in MIC between conditions, a Welch two-sample t-test was conducted between the SCFM group and each single nutrient medium.

Maximal growth rates (μmax) were determined using the splines function from the grofit package [26], fitted to growth curves (n = 3 biological replicates) for each evolutionary lineage. Growth measurements were recorded at regular intervals to ensure accurate curve fitting. Fold changes in μmax for antibiotic-evolved lineages were calculated relative to the corresponding antibiotic-free lineages in the same evolution medium. A Welch two-sample t-test, using the R base function, was used to compare the evolved lineages with the antibiotic-free controls evolved under the same medium condition.

To determine how mutation profiles differed between nutrient conditions, a partial least squares discriminant analysis (PLS-DA) was performed separately for each antibiotic using the mixOmics package [27]. The first four components were extracted to generate Variable Importance in Projection (VIP) scores, which were used to evaluate the distinguishability of mutated genes across nutrient conditions. VIP scores provided a measure of each gene's contribution to differentiation between nutrient environments, aiding in the identification of key mutations.

6.3. Results

Single nutrients conditions differentially shape antibiotic resistance evolution

We assessed the impact of specific single nutrient conditions on antibiotic resistance acquisition in *P. aeruginosa* PAO1. To this end we performed serial passaging under stepwise increasing antibiotic concentrations for five different antibiotics for a period of 10 days. *P. aeruginosa* PAO1 evolved in nutrient-rich SCFM medium was used as control. We evaluated changes in antibiotic susceptibility for the lineages under differential medium and antibiotic conditions through determination of the relative change in minimum inhibitory concentration (MIC) of SCFM-lineages in comparison to the parental strain (**Fig. 1**).

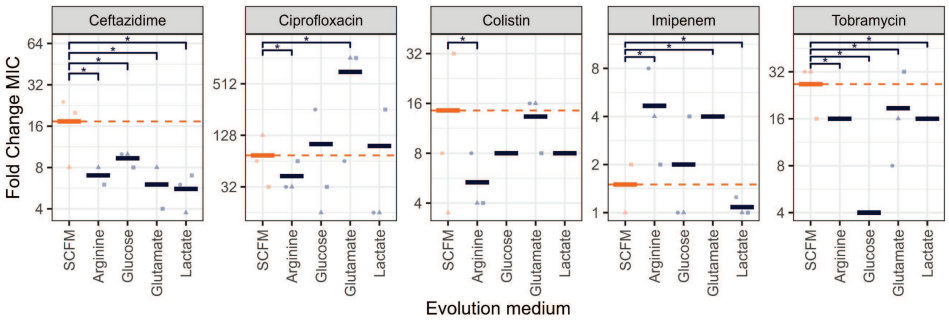

Figure 1. Change in minimum inhibitory concentrations (MICs) for five antibiotics in lineages evolved under different medium conditions. *P. aeruginosa* PAO1 strain was evolved in single nutrient media (arginine, glucose, glutamate, lactate) and synthetic cystic fibrosis sputum media (SCFM) as control, under incrementally increasing antibiotic concentrations. All MICs were determined in SCFM. Bold horizontal lines represent the mean fold change in MIC of the evolution condition relative to the parental strain. Different evolutionary lineages are indicated by shapes: (•) for replicate 1, (▲) for replicate 2, and (▪) for replicate 3. A Welch two-sample t-test was performed between the SCFM group and each single nutrient medium, with * indicating $p < 0.05$.

When considering the differential effects of media on resistance acquisition, for ceftazidime, the magnitude of MIC increase was consistently lower across all single nutrient media compared to SCFM, with limited differences between specific minimal media. For all four remaining antibiotics, substantial differences in MIC were found across single nutrient media. For ciprofloxacin, evolution in glutamate medium led to a substantially increased MIC, while the arginine condition results in a comparatively smaller MIC. Imipenem MICs only increased in arginine and glutamate medium, with no significant MIC change after evolution in SCFM, glucose, or lactate. For tobramycin, evolution in glucose medium resulted in substantially lower MICs compared to all other conditions. For colistin, the largest MIC increases were observed after evolution in SCFM and glutamate medium, while the smallest MIC decrease was noted in arginine. Across all antibiotics, it can be concluded that evolution in single nutrient media with arginine, glucose, and lactate often results in attenuated resistance acquisition, while resistance acquisition in glutamate media showed higher outcomes (**Fig. S1**).

Resistance evolution under single-nutrient conditions results in limited fitness changes

To evaluate if differences after evolution across single nutrient media could be explained by differences in fitness, we compared the maximal growth rate (μ_{max}) of all evolution lineages under antibiotic-free conditions in the original evolution media and SCFM. Overall, largest effects on μ_{max} were found when the original growth medium was used. Significant reductions in μ_{max} were observed for ceftazidime and tobramycin lineages evolved in glucose, and for ciprofloxacin lineages evolved in arginine and lactate (**Fig. 2**). These reductions were however absent for μ_{max} estimated derived in SCFM. The magnitude of changes in μ_{max} did not correlate with the changes in MIC in the evolution lineages (**Fig. S2**).

To further evaluate whether observed μ_{max} changes were antibiotic or medium dependent, we performed growth rate analysis of all evolution lineages across all conditions (**Fig. S3**). The observed reduction in μ_{max} for colistin- and imipenem-resistant lineages was consistent in glucose minimal media, regardless of the evolution media. Ciprofloxacin-resistant lineages generally exhibited reduced μ_{max} in minimal arginine media, except for those evolved in lactate. Notably, ciprofloxacin lineages evolved in lactate exhibited reduced

growth in all other single nutrient media, suggesting a unique metabolic adaptation or trade-off specific to lactate-driven resistance evolution.

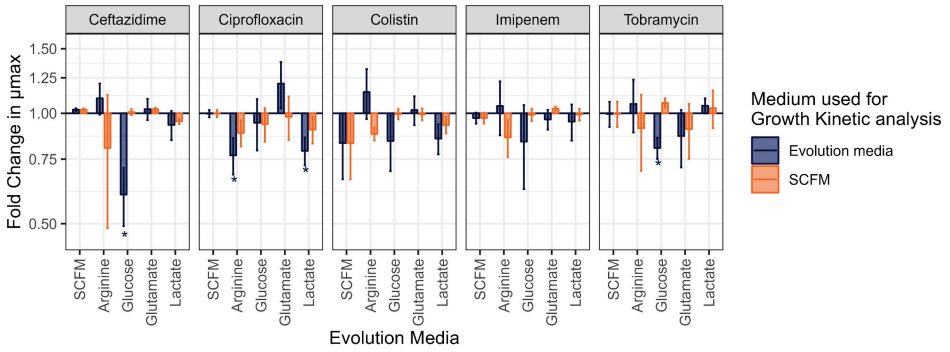

Figure 2. Changes in maximal growth rate of antibiotic evolved lineages upon re-culturing in their evolution medium and SCFM. The maximal growth rates (µmax) were determined by applying a spline function to triplicate growth curves obtained under antibiotic-free conditions. Fold changes are calculated relative to lineages without antibiotic in the corresponding medium. A Welch two-sample t-test was used to determine the difference between the evolved lineages and the antibiotic free controls evolved in the same medium (* indicating $p<0.05$).

Medium-specific genetic variations differentiate between evolution conditions.

Whole genome sequencing was performed for final evolution lineages for ciprofloxacin, colistin, and tobramycin to evaluate genetic variants across parallel evolved biological replicates (**Fig. 3**). Single nucleotide polymorphisms (SNPs), insertions, and deletions observed with a frequency higher than 20% and present within more than 1 lineage were included due to large heterogeneity within evolutionary lineages.

To determine how mutations differ between the evolution media, a partial least squares discriminant analysis (PLS-DA) was applied for each antibiotic. This analysis indicates the discriminatory importance of mutations that allow differentiation across specific medium conditions, quantified using a

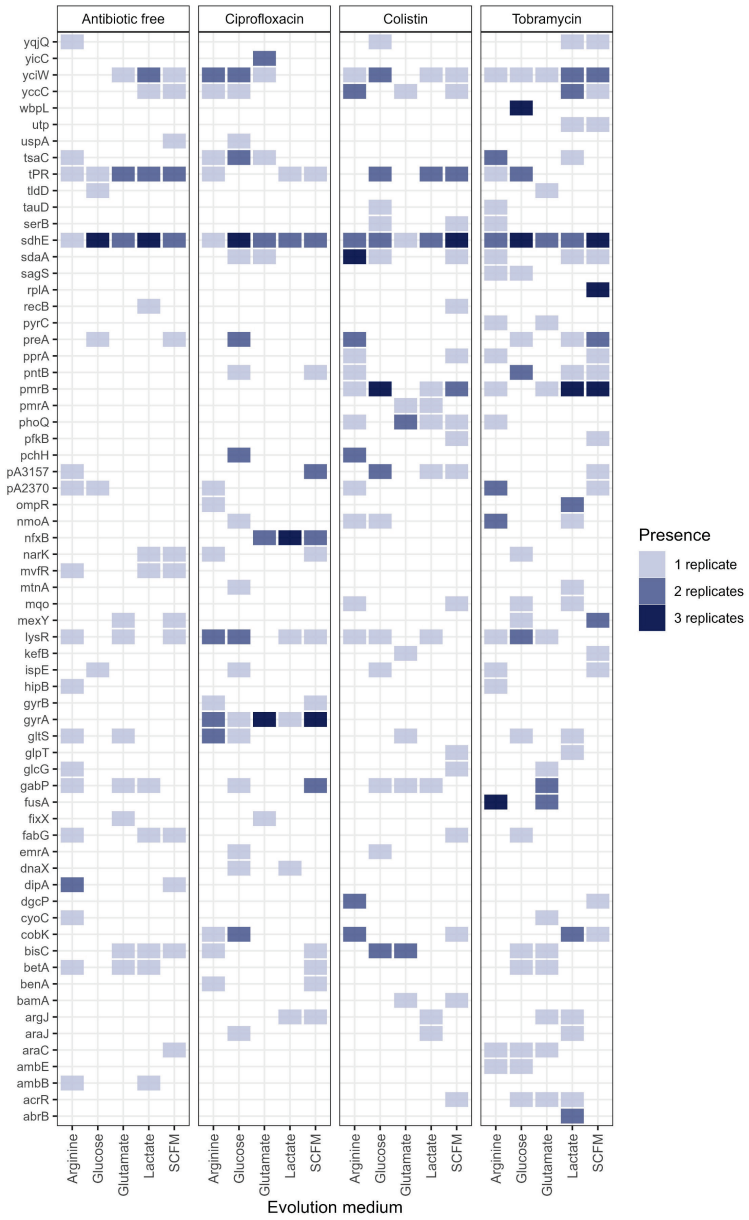

Figure 3. Whole-genome sequencing-derived mutations in evolution lineages from different antibiotic and nutrient conditions. The occurrence of single nucleotide polymorphisms (SNPs), deletions, and insertions associated with annotated genes are indicated. The color scale represents the number of evolutionary lineages under the same condition where a mutation was observed. All mutations detected only once are excluded from the figure.

variance importance in projection (VIP) score. Mutations in the top six genes ranked by VIP score were selected and visualized as potential mutations associated with media-specific adaptation for each antibiotic (**Fig. 4**). Among the top distinguishing genes, only three loci mutations appeared in a single evolution condition. Two were in tobramycin lineages: *rplA* for SCFM and *wbpL* for glucose medium (**Fig. 4A**). Other genes specific for tobramycin were mutations in the *pmrB* gene, which was mutated in all media except glucose, and the *fusA* gene, which was specific to arginine and glutamate minimal media. For ciprofloxacin, mutations in *yicC* were unique for the ciprofloxacin-glutamate condition, while mutations in *nfxB* were present in lineages evolved glutamate, lactate, and SCFM media (**Fig. 4B**). Finally, mutations in *bisC, cobK, dgcP, PA3157, preA, pchH,* and *sdaA* had high VIP scores for distinguishing evolution conditions when stratified by antibiotic, but were also present in various other media conditions across different antibiotics, including antibiotic-free evolution. The top VIP scores after colistin evolution consist only of these genes, indicating a limited presence of condition-specific mutations in colistin resistance (**Fig. 4C**).

6.4. Discussion

We demonstrated that *P. aeruginosa* can rapidly develop antibiotic resistance in single nutrient media, with the magnitude of MIC increase varying substantially between evolution conditions as well as antibiotics. Despite the MIC variation, changes in mutant growth rates (μ_{max}) were generally consistent among lineages evolved under the same antibiotic.

Across the single nutrient conditions, we found the smallest increase in MIC in lineages evolved for the beta-lactam antibiotics imipenem and ceftazidime. This may be explained by resistance development against imipenem requiring multiple mutation steps[28] and the limited duration of the evolution experiment. Previous findings demonstrated similar limited MIC increases for ceftazidime during evolution in minimal media conditions[29–31]. Lineages evolved during exposure to ciprofloxacin, colistin, and tobramycin, larger differences between MICs for different nutrient conditions were observed, which were further genetically characterized.

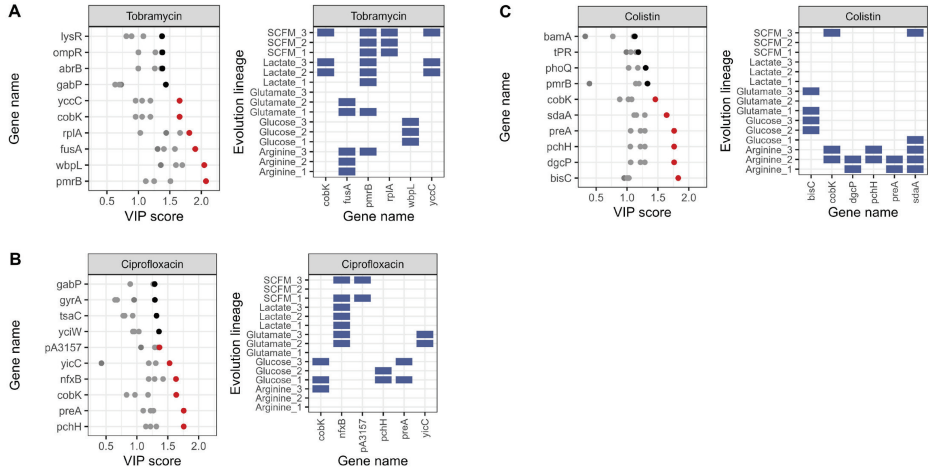

Figure 4. Key mutations distinguishing mutational profiles of culture conditions across (**A**) tobramycin, (**B**) ciprofloxacin and (**C**) colistin. (Left) VIP (Variable Importance in Projection) scores from a 4-dimensional partial least squares discriminant analysis (PLS-DA) highlight key mutations distinguishing culture conditions. The three lowest VIP scores for each mutation are colored grey, while the highest VIP score per drug is highlighted in black, and in red if identified as a key distinguishing mutation. (Right) Heatmaps display the six highest-scoring locus mutations, with colored tiles indicating mutation presence (x-axis) across evolutionary lineages (y-axis). These visualizations provide a comparative overview of mutation patterns across culture conditions and evolutionary lineages for the different antibiotics.

For ciprofloxacin lineages, the lowest MIC increases were found in arginine-lineages and the highest increases in glutamate-lineages. These differences may be explained due to differences in accumulation of specific established fluoroquinolone resistance mutations, particularly *gyrA* and *nfxB*[32]. The *gyrA* gene encodes a subunit of DNA gyrase, which is the primary target of fluoroquinolones, and mutations in this gene typically reduce the binding affinity of the antibiotic. Mutations in *nfxB* lead to overexpression of the MexCD-OprJ efflux pump, which exports various antibiotics. Whilst mutations in *gyrA* were common across all media, the glutamate-lineages with the highest MIC increases also carried *nfxB* mutations. We observed no changes in μ_{max} for these mutants, in line with previous reports for *P. aeruginosa* on compensatory effects

associated with *gyrA* mutations and *nfxB*-induced MexCD-OprJ efflux pump upregulation[19,33]. The burden of these compensatory effects could however still influence the evolutionary selection of these mutations. Ciprofloxacin-lineages demonstrated reduced μ_{max} in arginine medium, the condition in which this specific mutation combination was absent. The absence of *gyrA* and *nfxB* mutations in arginine-lineages, along with the lower μ_{max} of ciprofloxacin-lineages in arginine, suggests that metabolic constraints may hinder the establishment of these mutations under such conditions. In the case of *nfxB*-mediated upregulation of the MexCD-OprJ efflux pump, an active proton motive force (PMF) is required for export[34]. Arginine metabolism may lead to a reduced PMF, as energy can also be derived via the arginine deiminase pathway rather than PMF-generating oxidative phosphorylation[35]. Glutamate, by contrast, serves as a central metabolite in nitrogen metabolism and has been shown to reduce ciprofloxacin sensitivity in *P. aeruginosa*[36–38]. Glutamate has also been reported to enhance antibiotic penetration and counters efflux, thereby increasing intracellular ciprofloxacin concentration[39–41]. This combination raises the evolutionary pressure of ciprofloxacin in glutamate medium, which can result in faster and different acquisition of resistance[17]. We speculate that this pressure contributes to the unique *yicC* mutation observed in glutamate medium. Although less studied in *P. aeruginosa*, *yicC* in *E. coli* has been linked to DNA stress responses and RNA degradation[42,43], both processes linked to ciprofloxacin exposure.

Colistin resistance development showed considerable variation in MICs between lineages and across nutrient conditions. Similarly, no distinct patterns in mutations emerged across different nutrient conditions. Colistin resistance in *P. aeruginosa* primarily arises from lipid A modifications in the lipopolysaccharide (LPS) layer[44,45], with no observed fitness cost[45,46]. This aligns with our findings of increased MIC and unaffected μ_{max} in lineages carrying mutations in the lipid A regulatory genes *phoQ* and *pmrAB*. Interestingly, we observed a reduced μ_{max} when re-growing colistin-evolved lineages in glucose medium, except for those lineages originally evolved in glucose. Glucose is central to glycolysis and the pentose phosphate pathway, both of which supply precursors essential for the lipid A modifications regulated by *phoQ* and *pmrAB*[47]. *P. aeruginosa* possesses an inherently less efficient glycolytic pathway[24], and our findings suggest this

efficiency is further compromised if glucose utilization is not actively selected during colistin exposure. Under colistin exposure, *P. aeruginosa* has been suggested to utilize glucose as an osmotic regulator[48–50]. The fact that glucose-lineages exhibited a distinct phenotype, which could not be readily explained from high diversity in mutational profiles across all lineages, underscores the plasticity of colistin resistance in *P. aeruginosa*.

The evolution of tobramycin resistance exhibited significant variation in MIC across nutrient conditions. Substantial differences in mutational patterns among lineages were found, consistent with the extensive aminoglycoside resistome[51]. One key mutation observed was in *fusA*, a gene encoding elongation factor G, which is involved in ribosomal translocation. Mutations in the *fusA* gene have been identified in clinical isolates and are known to confer tobramycin resistance by altering ribosome function, the primary target of aminoglycosides[52]. Although these mutations are often associated with a fitness cost, they had not previously been linked to specific nutrient conditions. In our study, *fusA* mutations were observed exclusively in lineages evolved in arginine and glutamate media. One possible explanation is the known enrichment of *fusA* mutations in biofilm-grown populations[53]. This may be relevant given the reduced motility of *P. aeruginosa* in arginine, a phenotype commonly associated with biofilm formation[54]. Additionally, *fusA* mutations are known to influence quorum sensing, particularly through interaction with *lasR*, a quorum sensing regulator that increases aminoglycoside resistance[55]. Since glutamate nitrogen metabolism plays a critical role in quorum sensing pathways in other species[56,57], this may suggest a metabolic link between glutamate availability and selection for *fusA* mutations during tobramycin exposure. The *fusA* mutations co-occurred twice with *pmrB* mutations but did not lead to significant MIC changes to single mutations. The *pmrB* gene encodes a sensor kinase involved in LPS modification, a mechanism linked to both increased tobramycin resistance and increased susceptibility in some contexts[58,59]. The highest increase in tobramycin MIC was observed when *pmrB* mutation was accompanied with an SCFM-specific *rplA* mutation. The rplA gene is involved in ribosome assembly, and is known to reduce aminoglycoside binding and induce efflux pump overexpression in other species[60,61]. The combination of *pmrB-rplA* mutation seemed to be specific to nutrient rich conditions, yet there is no direct link between these mutations and

nutrient conditions in the literature. The *pmrB* mutations were found in lineages evolved in all nutrient conditions except glucose. Similar to the observed fitness cost of LPS-modifying mutations under colistin pressure in glucose medium, the absence of *pmrB* mutations in glucose-evolved lineages may be related to the altered and less efficient glycolytic pathway of *P. aeruginosa*[24]. Instead, glucose-evolved lineages harbored mutations in *wbpL*, another gene essential for LPS biosynthesis. Alteration of *wbpL* likely reduces aminoglycoside uptake[51,62]. Consistent with this, we observed no changes in μ_{max} across all tobramycin lineages. This demonstrates that, even in the absence of significant fitness costs, nutrient-induced adaptations favor distinct mutational patterns, as the importance of cellular processes related to tobramycin activity varies between conditions. These findings align with previous observations showing that LPS-modifying mutations are common in sessile biofilm populations, whereas transcriptional regulator mutations dominate in planktonic populations[53]. Together, our data support the conclusion that nutrient environments strongly influence both the metabolic state and lifestyle of *P. aeruginosa*, thereby shaping the adaptive pathways used to acquire tobramycin resistance.

To our knowledge, nutrient-specific differences in resistance evolution have not been explored beyond this study, although several studies have examined the impact of nutrient-rich versus nutrient-poor environments on antibiotic resistance development[18–21,29,63]. In this light, for ceftazidime and tobramycin, we observed a significantly higher MIC increase in nutrient-rich SCFM compared to the single nutrient conditions. For colistin, ciprofloxacin, and imipenem, such differences were less evident, with only ciprofloxacin evolution in glutamate minimal medium resulting in a significantly higher MIC compared to SCFM. Overall, our findings highlight the importance of carefully considering medium nutrient composition during in vitro experimental evolution studies.

In this study, all MIC testing of evolved lineages was conducted in SCFM. This approach enabled direct comparisons of permanent phenotypic and genotypic changes among evolved lineages, as confirmed through sequencing data. Conducting MIC assays in SCFM rather than in the specific evolution media resulted in the loss of a phenotypic dimension related to nutrient-specific antibiotic sensitivity adaptations. Although addressing this additional complexity was beyond the scope of the current study, changing nutrient conditions are

known to significantly influence antibiotic sensitivity[13,16]. Consequently, our results may underestimate the full spectrum of phenotype variability, further underscoring the importance of identifying the biochemical mechanisms driving antibiotic sensitivity changes. The use of -omics approaches offers valuable insights into how cells adapt to different environments during antibiotic exposure. For instance, metabolomics studies would be relevant to further evaluate changes in cellular energy metabolism or metabolic rewiring that support membrane modifications[64], whereas proteomics has been employed to unravel a broad range of cellular adaptations[65]. By integrating these system-level analysis with extensive genotypic data[66], we can better understand the nutrient-specific differences in phenotype-genotype relationships observed between laboratory findings and clinical isolates. Multiple studies in other pathogens have demonstrated that adjusting media composition to better mimic in vivo conditions leads to in vitro antibiotic responses more closely mirroring clinical observations[67–71], underscoring the importance of environmental specific conditions to enhance clinical relevance of ALE.

We employed a stepwise increase in antibiotic concentration to rapidly drive resistance evolution over short timescale[72], enabling us to investigate a broad set of nutrient-antibiotic combinations. While this strategy provides valuable insights, additional confirmatory experiments with increased replicates and extended evolution periods would offer a more comprehensive view of how nutrients influence evolutionary trajectories in the long run. In clinical settings, antibiotic concentrations are highly dynamic, whereas nutrient profiles, though variable across infection sites, tend to remain relatively stable within the larger volumes of human tissues and fluids[11,73]. Building on our findings from stepwise adaptation studies, future work could leverage continuous culture systems to better control nutrient levels and mimic realistic antibiotic concentration profiles[74,75].

Overall, our findings demonstrate that evolution in single nutrient conditions results in significant variation in antibiotic resistance acquisition in *P. aeruginosa*. By focusing on multiple antibiotics and nutrient environments, we showed that the effects are specific to combinations of antibiotics and nutrients. These findings lay the groundwork for broader incorporation of nutrient composition as a key factor in antibiotic resistance evolution studies. Understanding the mechanisms behind these nutrient-induced differences is an important step toward unraveling the complex evolutionary trajectories seen in the CF lung.

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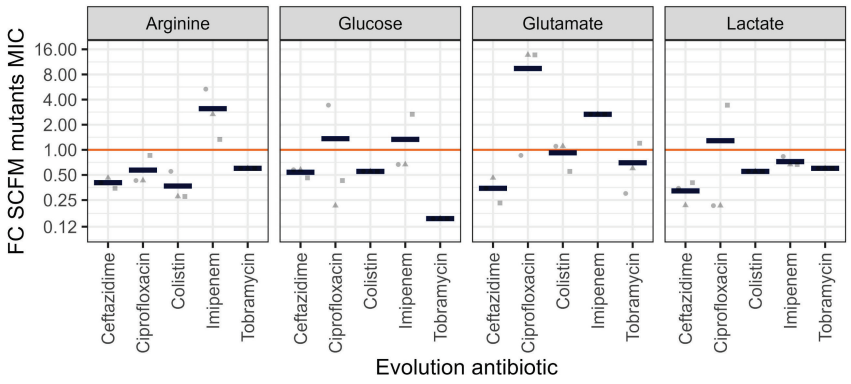
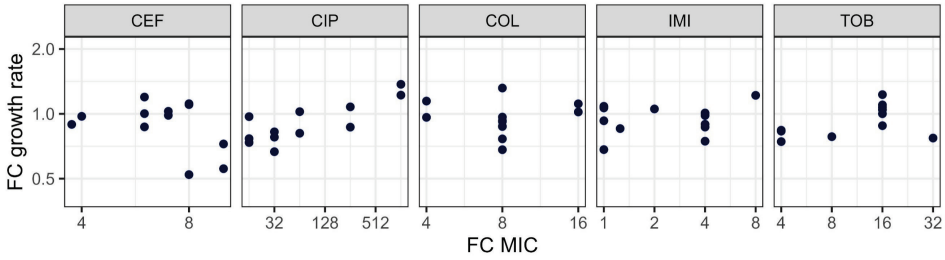
6.7. Supplementary Materials

Supplemental table 1. Detailed content list of synthetic cystic fibrosis sputum media

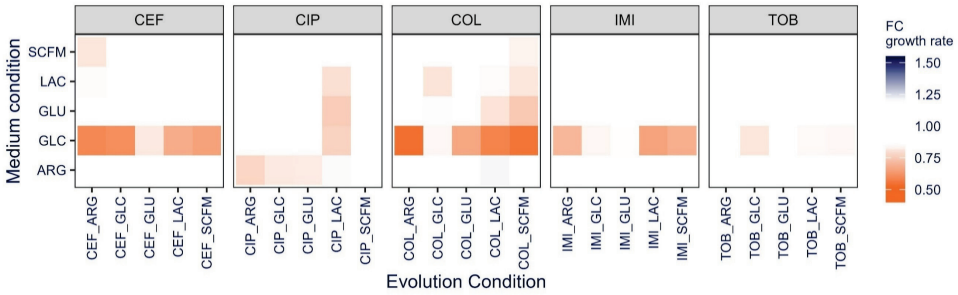
	Name	Concentration (mM)	Company information
M9 buffer	di-sodium hydrogen phosphate (Na_2HPO_4)	90.2	Thermo Fisher Scientific
	Potassium di-hydrogen phosphate (KH_2PO_4)	22.0	VWR International
	Sodium chloride (NaCl)	8.5	Merck KGaA (Avantor™)
	Ammonium chloride (NH_4Cl)	18.6	Alfa Aesar
	Magnesium sulphate hepta-hydrate ($MgSO_4$)	1.0	VWR International
	Calcium chloride ($CaCl_2$)	0.1	Acros Organics
Salts	Potassium nitrate (KNO_3)	0.35	Acros Organics
	Iron sulphate ($FeSO_4$)	0.0036	Alfa Aesar
Suppl.	BME Vitamin solution	*1x*	Thermo Fisher Scientific
Trace metals	Di-sodium Ethylene di-amine tetra-acetic acid (EDTA)	0.002 (mg/mL)	J.T. Baker (Avantor™)
	Zinc Sulphate hepta-hydrate ($ZnSO_4$)	0.23 (mg/mL)	Alfa Aesar
	Boric acid (H_3BO_3)	0.111 (mg/mL)	Acros Organics
	Manganese chloride tetra-hydrate ($MnCl_2$)	0.051 (mg/mL)	Sigma Aldrich (Avantor™)
	Cobalt chloride ($CoCl_2$)	0.017 (mg/mL)	Alfa Aesar
	Copper Sulphate penta-hydrate ($CuSO_4$)	0.015 (mg/mL)	Sigma Aldrich (Avantor™)
	Ammonium hepta-molybdate tetra hydrate ($(NH_4)_6\ Mo_7O_2$)	0.01 (mg/mL)	Alfa Aesar
Nutrients	Alanine (Ala)	1.8	Chem-Impex International
	Arginine (Arg)	0.3	Chem-Impex International
	Aspartate (Asp)	0.8	Chem-Impex International
	Cysteine (Cys)	0.2	Chem-Impex International
	Glucose (GLC)	3.2	Alfa Aeser
	Glutamate (Glu)	1.5	Chem-Impex International
	Glycine (Gly)	1.2	Acros Organics
	Histidine hydrochloride (His)	0.5	Chem-Impex International
	Isoleucine (Ile)	1.1	Chem-Impex International
	Lactate (LAC)	9.0	Biosynth International
	Leucine (Leu)	1.6	Chem-Impex International
	Lysine hydrochloride (Lys)	2.1	Thermo Fisher Scientific
	Methionine (Met)	0.6	Chem-Impex International
	Phenylalanine (Phe)	0.5	Chem-Impex International
	Proline (Pro)	1.7	Thermo Fisher Scientific
	Serine (Ser)	1.4	Chem-Impex International
	Threonine (Thr)	1.0	Chem-Impex International
	Tryptophan (Trp)	0.01	Chem-Impex International
	Tyrosine (Tyr)	0.8	Chem-Impex Internationa
	Valine (Val)	1.1	Chem-Impex International



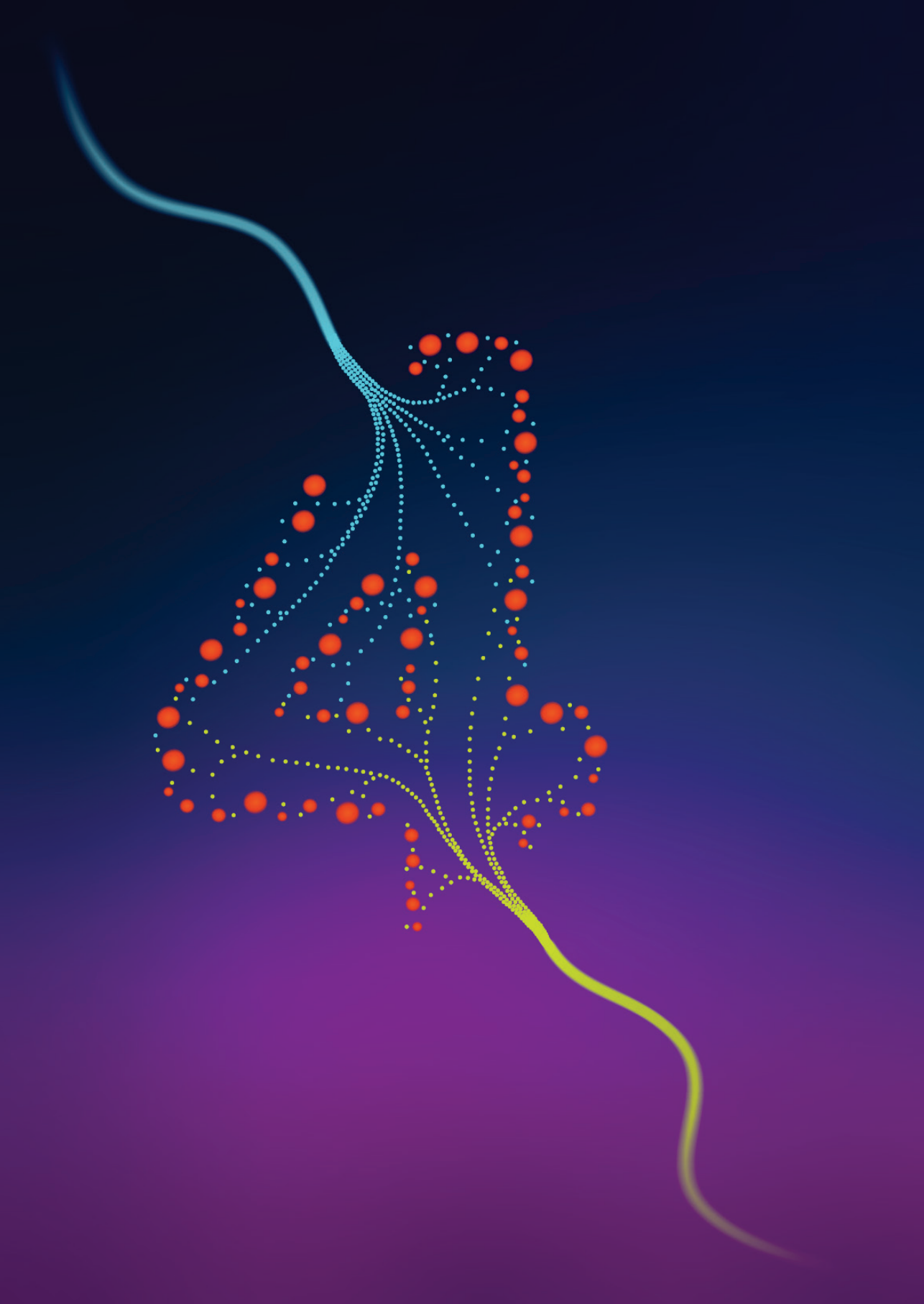
Supplemental figure 1. Comparison of minimum inhibitory concentrations (MIC) between lineages evolved in single-nutrient media and nutrient-rich synthetic cystic fibrosis sputum medium (SCFM). *P. aeruginosa* PAO1 was evolved in single-nutrient media (arginine, glucose, glutamate, lactate) with incrementally increasing antibiotic concentrations. The difference in evolution conditions is shown as the fold change in MIC of lineages evolved in single-nutrient media relative to lineages evolved in SCFM. All MICs were determined in SCFM. Bold horizontal lines indicate the mean fold change in MIC of the SCFM lineages. Symbols represent distinct evolutionary replicates: (•) replicate 1, (▲) replicate 2, and (▪) replicate 3.



Supplemental Figure 2. Correlation between the maximal growth rate (μmax) and minimal inhibitory concentration (MIC) of evolved *P. aeruginosa* PAO1 lineages. Maximal growth rates were determined by spline fitting of triplicate growth curves under antibiotic-free conditions and expressed as fold changes relative to lineages evolved without antibiotic in the same medium. MICs were measured for lineages evolved in single-nutrient media under incrementally increased concentrations of ceftazidime (CEF), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), or tobramycin (TOB). MIC fold changes are shown relative to the parental strain.



Supplemental figure 3. Changes in the maximal growth rates of antibiotic-evolved lineages after re-culturing in antibiotic-free media. Lineages were obtained by incrementally increasing concentrations of five antibiotics (ceftazidime (CEF), ciprofloxacin (CIP), colistin (COL), imipenem (IMI), and tobramycin (TOB)) under various nutrient conditions. Maximal growth rates were determined by spline fitting of triplicate growth curves in antibiotic-free synthetic cystic fibrosis sputum medium (SCFM) or in single-nutrient media (arginine (ARG), glucose (GLC), glutamate (GLU), lactate (LAC)). Fold changes are shown relative to lineages evolved under antibiotic-free conditions in each respective medium.



Section IV

Discussion and summary

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 7

General discussion, future perspectives, and and overall conclusion

7.1. Summary and general discussion

Pseudomonas aeruginosa is classified by the World Health Organization as a top-priority pathogen, owing to its capacity for severe infections and its resistance to many antimicrobials. This threat is particularly acute for immunocompromised patients, such as those with cystic fibrosis (CF), who often acquire chronic P. aeruginosa infections early in life. Prolonged antimicrobial exposure in these infections drives the development of multidrug-resistant strains. To combat this rise in resistance, it is increasingly recognized that understanding how P. aeruginosa survives within the CF lung is crucial. In this context, a key element lies in the infectious microenvironment, which shapes bacterial physiology and influences antibiotic efficacy1. In an effort to capture the influence of the infectious microenvironment, recent studies have employed innovative in vitro culture media to better replicate the infectious phenotype. However, the diverse microenvironments in the CF lung, coupled with the biological versatility of P. aeruginosa, call for a more comprehensive approach to elucidate its triangular relationship with antibiotic pharmacodynamics (PD). This thesis hypothesized that nutrient conditions in the environment play a central role as pharmacodynamic drivers and key determinants in the evolution of antibiotic resistance, ultimately affecting therapeutic outcomes. To address this, foundational work was conducted to elucidate how altered nutrient and oxygen conditions influenced the antibiotic pharmacodynamics of P. aeruginosa.

In Section I, a comprehensive overview of the diverse microenvironments within the CF lung was provided and the ways in which *P. aeruginosa* adapts to the available nutrients were described (**Chapter 2**). This thesis highlights that these microenvironments can vary among patients and even within the same lung, potentially affecting *P. aeruginosa* adaptation in distinct ways. To explore this in more detail in Section II, the impact of specific nutrients (**Chapter 3**) and oxygen gradients (**Chapter 4**) relevant to the CF lung on antibiotic sensitivity was investigated. In Section III, the role of microenvironmental interactions in driving evolutionary processes by influencing the selection of mutants was examined. It was discussed how exploiting metabolic adaptations during resistance evolution may offer therapeutic advantages (**Chapter 5**), and *P.*

aeruginosa adaptation during antibiotic resistance development was reported under single-nutrient conditions (**Chapter 6**).

Adaptation to heterogeneous cystic fibrosis lung

In **Chapter 2**, the nutrient environments present in the CF lung were reviewed for its role in shaping the metabolic adaptation of *P. aeruginosa*, as these adaptations play a key role in the failure of antibiotic therapies. The bacterial metabolic state determines bacterial growth, redox balance, and energy levels, all factors that influence antibiotic susceptibility. In this context, the thick, dehydrated mucus in the CF lung creates diverse microenvironments characterized by nutrient-rich but oxygen-limited conditions. These gradients support the formation of biofilm aggregates by *P. aeruginosa*, which further compartmentalize nutrients and oxygen, shaping metabolic heterogeneity. The metabolic versatility of *P. aeruginosa* is central to its survival and adaptation within the CF lung environments. *P. aeruginosa* can utilize a wide range of substrates, including amino acids and short-chain fatty acids, and flexibly switch between aerobic respiration, fermentation, and denitrification to maintain energy production under varying conditions[2].

Metabolic adaptations strongly influence antibiotic sensitivity. Antibiotics such as aminoglycosides, fluoroquinolones, β-lactams, and polymyxins all depend on specific metabolic states for their effectiveness[3]. *P. aeruginosa* can rewire its metabolism by suppressing respiration, activating metabolic shunts, or producing protective extracellular matrix components, thereby reducing the impact of these drugs.

Importantly, the CF lung microenvironment is not uniform. Intra- and inter-patient variability shaped by factors like lung compartmentalization[4], CF genotype[5], comorbidities[6] leads to significant differences in nutrient availability and thus in antibiotic response. Understanding these metabolic responses in physiologically relevant contexts is a critical step toward more effective, personalized antibiotic interventions. Progress depends on integrating clinical insights with advanced *in vitro* models and multi-omics approaches to accurately reflect the complex CF lung environment[7]. Overall, this chapter outlined the current understanding on this topic and identified important gaps to guide future research.

Nutrients shape antibiotic treatment response

Nutrients and oxygen are among the most evident drivers of metabolic processes, which have increasingly been linked to the mechanisms of action of multiple antibiotic classes[8,9]. As a result, reduced antibiotic sensitivity is often associated with nutrient-scarce or oxygen-deprived environments, and supplementation is sometimes used to sensitize pathogens[10]. Although these observations highlight the regulatory role of the nutrient environment in shaping microbial responses to antibiotics, the impact of specific conditions relevant to the CF lung remains poorly understood.

In **Chapter 3**, the impact of specific nutrients antibiotic sensitivity was investigated. To this end, a basal culture medium was modified by alternately adding individual nutrients, and changes in antibiotic response were measured using time-kill assays with a bioluminescent *P. aeruginosa* strain, allowing real-time monitoring of bacterial population dynamics.

Using mathematical PD modeling, changes in antibiotic responses across different nutrient conditions were assessed. By focusing on quantification of changes in the half-maximal effective concentration (EC_{50}), nutrient-induced, antibiotic class-specific changes in antibiotic PD were revealed, indicating distinct underlying biochemical mechanisms. This finding expands beyond the traditional view of reduced metabolic activity in nutrient-poor conditions typically correlated with antibiotic tolerance or persistence mechanisms[3]. The absence of significant fitness differences among bacterial populations in our media formulations further suggests that biochemical, rather than purely growth-rate dependent adaptations, underly the observed sensitivity shifts. This aligns with a previous mathematical model, showing a stronger correlation between antibiotic lethality and metabolic states rather than growth rates alone[11].

Investigating the impact of single-nutrient alterations provides mechanistic clarity by establishing a direct relationship between specific metabolic pathways and antibiotic responses. These single-nutrient effects were shown to have clinical relevance, as illustrated by our in vitro pharmacokinetic-pharmacodynamic (PK-PD) simulations showing nutrient-induced changes in treatment outcomes. Previous studies have similarly shown the potential for leveraging nutrient-based findings to identify adjuvant therapies, combining

antibiotics with nutrient modulation to enhance bacterial killing10. While it is important to acknowledge the inherent limitations of our in vitro models, since antibiotic responses in clinical environments involve a broader and more complex range of interactive factors, our results nonetheless represent meaningful progress. Specifically, these findings advance our understanding of the personalized nature of treating P. aeruginosa infections, which are heavily influenced by microenvironmental interactions.

In addition to nutrient availability, oxygen levels represent another key environmental factor that is often overlooked in conventional in vitro antimicrobial activity assays. In **Chapter 4**, the specific impact of oxygen availability on antibiotic responses in P. aeruginosa was investigated. Instead of focusing solely on acute responses to anoxia, the study also assessed how prolonged anoxic exposure (<1% oxygen for 22 days) impacts antibiotic sensitivity. To this end, time-kill assays were performed, comparing a laboratory strain of P. aeruginosa to lineages adapted through prolonged growth under anoxic conditions.

The results indicated that antibiotic effects under anoxic conditions are antibiotic-specific, consistent with known differences in oxygen dependency among antibiotic mechanisms of action reported previously[12–14]. The antibiotic sensitivity profiles changed profoundly following prolonged anoxic conditioning, suggesting sustained modifications in cellular processes related to antibiotic sensitivity. Importantly, these differences sustained when anoxically adapted strains were subsequently cultured under atmospheric conditions, underscoring stable physiological adaptations.

The distinct antibiotic sensitivity profiles observed following anoxic adaptation highlight the potential limitations of standard *in vitro* antimicrobial activity assays, which do not accurately replicate conditions of anoxic infection sites. This emphasizes the urgent need to develop culture conditions that better mimic *in vivo* infection environments. Additionally, the findings suggest that brief exposure to clinically relevant conditions is insufficient. Instead, a comprehensive characterization encompassing multiple, sustained environmental conditions is required to reflect the true complexity of clinical infections. Although this study initially focused on conditions relevant to cystic fibrosis (CF), similar considerations likely apply to other *P. aeruginosa* infection

sites, such as urinary tract infections[15], mucus plugs in chronic obstructive pulmonary disease (COPD) or bronchiectasis[16].

Overall, the findings in Section II highlight the critical role of metabolic regulation in determining antibiotic sensitivity of *P. aeruginosa* under diverse nutrient and oxygen conditions. Nutrients and oxygen form the foundational components for cellular biosynthesis and energy metabolism. Thus, their influence on antibiotic effectiveness might logically follow, since antibiotics often target actively dividing cells. However, the results demonstrate that antibiotic sensitivity can be substantially altered by even single-nutrient changes, with specific outcomes dependent on the precise combination of nutrient conditions and antibiotic class.

The observations across various antibiotic classes support the concept that increased nutrient availability does not necessarily translate into enhanced antibiotic efficacy. Research on the metabolic-targeting natural product promysalin, which can exhibit both synergistic and antagonistic effects when combined with standard antibiotics in *P. aeruginosa*[17], underscores that nutrient metabolism can activate protective cellular pathways that counteract antibiotic actions. This indicates that nutrient metabolism can indeed trigger protective cellular pathways dependent on the antibiotic pressure[18]. It is also important to acknowledge that *P. aeruginosa* harbors an efficient hierarchical nutrient-utilization regulatory systems in nutrient-rich conditions, which means that combining separate nutrient media formulations will likely not produce additive antibiotic sensitivity effects. For example, *P. aeruginosa* can produce redox-active metabolites that suppress respiration and induce a low-energy cellular state even in oxygen-rich conditions[19]. These nutrient-induced metabolic changes result in phenotypic adapted strains over long-term colonization of the CF lung[20]. The findings in this section demonstrated that these adaptation processes profoundly impact antibiotic sensitivity profiles. Such insights underscore the importance of elucidating the biochemical basis of nutrient-driven antibiotic sensitivity and highlight the need for integrative, phenotype-focused -omics approaches. Metabolomics and proteomics are powerful techniques that can capture subtle shifts in intracellular metabolite concentrations, enzyme levels, and metabolic flux patterns, thereby providing a high-resolution view of the metabolic reconfigurations during antibiotic treatment[21,22].

If we are to adopt a more biochemically informed approach, it is crucial that future studies be conducted in environments representative of both the antibiotic's mechanism of action and the conditions of actual infections. In addition to the incremental approach of distinguishing the effects of individual nutrients, detailed investigation into the nutrient composition of the infectious environment is essential. The infectious microenvironment is dynamically shaped by interactions with host cells and microbial communities, generating metabolic byproducts and oxidative stress that modulate bacterial metabolism. Consequently, the use of co-culture systems that model host-pathogen[23] or polymicrobial interactions[24] is essential for fully capturing the intricate interplay between nutrient environments and antibiotic sensitivity. By building on this experimental data, future research can more accurately account for the complex nutritional and metabolic landscapes which *P. aeruginosa* exploits to influence antibiotic susceptibility.

Nutrients shape antibiotic resistance evolution

It is well established that phenotypic changes play a central role in the adaptive capacity of populations, driving natural selection. In this context, populations that adapt to environmental perturbations with higher fitness levels come to dominate. Under antibiotic pressure, heritable changes, such as chromosomal mutations that confer resistance through target modifications, reduced uptake, or increased active efflux, rapidly prevail. These changes are often integral to essential cellular mechanisms and, consequently, frequently incur a fitness cost. Beyond the fact that alterations in fitness and resistance mechanisms induce metabolic changes, it is increasingly recognized that mutations affecting metabolism represent a key category of antibiotic resistance mechanisms[25].

In **Chapter 5**, metabolic adaptations during antibiotic resistance evolution were reviewed, and it was discussed how metabolomics can be applied to understand and exploit these adaptations. Although metabolomics is the comprehensive study of all small molecules in a biological sample, there is no single analytical method that can measure them all due to their extensive physicochemical diversity. Therefore, effective metabolomics studies require thoughtful selection of analytical approaches and sample preparation methods that are tailored to the specific biological questions at hand. For example, the role of metabolic quenching was discussed to counter the high turnover rate of energy

metabolism; apolar extraction procedures were highlighted for retrieving membrane profiles for studying cell envelope changes; and spatially oriented ionization techniques were noted for their utility in examining cell-to-cell communication within biofilm structures. Although 23 metabolomics-focused papers were included in the review, these studies were distributed across various bacterial species and antibiotic classes. It can therefore be concluded that a more systematic application of metabolomics is needed to further elucidate the role of metabolism in antimicrobial resistance mechanisms.

In **Chapter 6**, the role of metabolism in antibiotic resistance evolution was further explored by challenging the metabolic versatility of *P. aeruginosa* through antibiotic adaptive laboratory evolution experiments conducted under single-nutrient conditions. This work was built on the concept that the metabolic constraints imposed by antibiotic resistance mutations, together with the regulatory influence of nutrient supply, shape the evolutionary response[26,27]. Phenotypic and genotypic changes in *P. aeruginosa* were investigated following a 10 day evolution period under antibiotic pressure, using high concentrations of arginine, glucose, glutamate, and lactate as single-nutrient conditions.

The evolved lineages displayed significant differences in minimal inhibitory concentrations (MIC) across the single-nutrient evolution conditions. Earlier work also reported nutrient-dependent MIC shifts in *P. aeruginosa*, but primarily when comparing nutrient-rich with nutrient-poor media[28]. Although that study found only modest MIC divergence, it still identified medium-specific resistance mutations. In our experiment, mutations unique to specific evolution conditions were also detected, but only six across the 15 distinct conditions that were sequenced. An additional partial least-squares discriminant analysis (PLS-DA) revealed a small set of mutational patterns that reliably distinguished our evolution conditions. The narrow range of mutational change parallels the almost identical post-evolution growth-rate shifts observed within each antibiotic class. This limited fitness variation steers the populations toward a restricted set of genotypes[29].

These findings demonstrated that even in single-nutrient media *P. aeruginosa* can evolve along multiple trajectories. However, this diversity manifests primarily as shifts in MIC rather than as distinct mutational signatures. Because *P. aeruginosa* can persist in the CF lung for years, the phenotypic changes observed may eventually solidify into stable genotypic differences. Continuous,

automated *in vitro* systems are well suited to study such long-term evolution[30], but they are technically demanding, time-consuming, and resource intensive. A more practical first step would be to investigate in more detail how infection-relevant nutrient conditions shape antibiotic resistance development, bearing in mind that altering even a single nutrient can tip the evolutionary balance.

In summary, Section III highlighted that metabolic processes play a crucial role in antibiotic resistance development and demonstrated that, despite its metabolic versatility, nutrients exert a regulatory influence on *P. aeruginosa* resistance development. Although our findings delve into how the nutrient environment contributes to the complex evolutionary trajectories observed in the CF lung, they represent only a small piece of the overall picture, and further research in this area remains essential. Nevertheless, the results indicate that the influence of the nutrient environment is antibiotic-dependent, underscoring the need for more in-depth investigation of the biochemical responses and specialization processes that occur during antibiotic exposure.

7.2. Future perspectives

Antibiotics are indispensable for everyday healthcare, yet immunocompromised patients bear a disproportionate burden of infectious disease and also serve as a reservoir for antibiotic-resistant strains. Examining infections in these individuals provides an unique window into the various ways antibiotic therapies can fail. The CF lung represents such an unique environment, where a typically commensal bacterium like *P. aeruginosa* becomes one of the most challenging infections to treat. Understanding the mechanisms that make the combination of the CF lung and *P. aeruginosa* so burdensome may also offer critical insights into other bacterial infections. A key factor shaping *P. aeruginosa* infection in the CF lung is the unique nutrient environment, yet this aspect is often overlooked. The nutrient environment can profoundly influence bacterial behavior and can lead to misinterpretation of antibiotic susceptibility testing performed using standard culture media. Therefore, in the quest to better understand the *P. aeruginosa* pathophysiology, it is essential to focus on: I) mapping physiologically relevant infection sites, II) examining the influence of nutrients on antibiotic

susceptibility testing, III) working on capturing nutrient-induced antibiotic sensitivity changes, and IV) elucidating the underlying biological mechanisms responsible for the antibiotic effect changes.

Mapping of physiologically relevant infection sites

The respiratory tract is a spatially organized system comprising diverse microenvironments, each of which may support distinct infection dynamics[31]. The findings presented in this thesis, demonstrating the strong influence of nutrient availability on antibiotic pharmacodynamics, underscore the critical need to improve our understanding of the chemical composition of these distinct niches. Accurate mapping of these environments is therefore an essential next step in this line of research.

It is crucial to recognize that not all samples taken from the CF lung are created equal and each sample carries inherent biases and limitations that influence interpretation. For instance, expectorated sputum is the most accessible and commonly used sample type, but it represents a heterogeneous blend of secretions from multiple regions of the lung. This pooling of distinct microenvironments reduces spatial resolution and contributes to a substantial disconnect between the chemical profiles observed in sputum and those derived from *in vitro P. aeruginosa* cultures[32]. Sampling epithelial lining fluid via microsamplers offers more region-specific insights but is limited by its invasiveness and the need for saline instillation, which dilutes the sample and complicates direct metabolite comparisons across patients or timepoints[33,34]. Another layer of complexity arises from the heterogeneous biofilms. Spatially resolved analytical techniques have revealed intricate metabolic structuring within biofilms, including mathematical models informed by -omics data that demonstrate their metabolic heterogeneity[35].

The field of oncology has pioneered efforts to address such complex biological heterogeneity through network-based frameworks. Over the past decade, researchers have begun constructing multiscale “tumor atlases” that integrate cellular phenotypes, bulk -omic alterations, and interactions with the tumor microenvironment[36]. These atlases are built by combining conventional pathology with spatial biology and multi-omic datasets, offering a comprehensive view of tumor evolution shaped by local conditions[37]. Beyond advancing

fundamental understanding, these tools now support stratification of patient populations and guide personalized treatment strategies.

Inspired by these advances in oncology, future efforts to characterize the infectious microenvironment of the CF lung must similarly embrace integrative, spatially resolved frameworks. This means not only aligning molecular, microbial, and pathological data layers, but also actively distinguishing between different CF patient phenotypes. Stratifying infections based on microenvironmental features will be essential for translating nutrient-induced pharmacodynamic effects into meaningful, individualized treatment decisions.

In vitro nutrient composition in antimicrobial activity assays

While our understanding of the spatial and chemical complexity of the infectious environment advances, accurately measuring its clinical impact requires that *in vitro* antimicrobial activity assays be adapted accordingly. The physicochemical composition of the culture medium represents a critical and tractable factor for more accurately mimicking infectious conditions[38]. Prior studies have shown that medium composition can significantly alter antibiotic activity[39], and findings presented in **Chapter 3** demonstrate that even a single nutrient modification can markedly shift antibiotic sensitivity. Combined with the environmental heterogeneity outlined previously, these results underscore the need to adapt *in vitro* antimicrobial assays to infection-specific microenvironments to better predict clinical outcomes and guide therapy.

Nutrient conditions are also likely to influence other *in vitro* antimicrobial activity assays, including combination antibiotic testing and biofilm susceptibility testing. For instance, the synergy of antibiotic combinations depends on drug-specific mechanisms of action[40], which can be sensitive to medium composition[41]. In biofilm models, nutrients critically impact maturation. In the CF lung, *P. aeruginosa* grows in aggregates that can be mimicked using alginate beads, but mature biofilms represented by full bead coverage require supplementation with alternative electron acceptors[42]. The reduced antibiotic susceptibility of biofilm populations are closely linked to the metabolic adaptations associated with their heterogeneous structure[43]. Incorporating infection-relevant nutrient environments into these alternative *in vitro* assays may be essential to overcome the inconsistent clinical outcomes they currently produce[44,45].

Completely changing the *in vitro* experimental methodologies for clinical antimicrobial susceptibility testing (AST) is challenging, as current standardized protocols ensure interlaboratory comparability and are far less time-consuming than alternative approaches. Another potential step in improving AST is refining isolate selection by extracting bacterial populations from different lung regions of the same patient, as regional adaptations may significantly influence antibiotic susceptibility[46]. For example, **Chapter 4** shows that oxygen levels affect antibiotic sensitivity, highlighting how microenvironmental specialization drives distinct colony phenotypes. A well-known CF-relevant subpopulation, small colony variants (SCVs), can survive otherwise lethal antibiotic concentrations but are often underrepresented in conventional AST due to their difficult-to-culture nature[47]. Moreover, as demonstrated in **Chapter 4**, specialization to anoxic conditions can lead to different treatment outcomes during aerobic *in vitro* experiments. To accurately assess antibiotic susceptibility in such specialized populations, AST must replicate the infection conditions under which isolates evolved. In this context, incorporating anaerobic AST may be particularly valuable for chronic *P. aeruginosa* infections.

In summary, accurately predicting antibiotic efficacy in the heterogeneous CF lung environment will require antimicrobial assays that incorporate relevant nutrient conditions, assay parameters, and isolate selection.

Nutrient induced changes to antibiotic pharmacodynamics

To map how nutrient availability modulates antibiotic PD, this thesis moved beyond static MIC testing and applied dynamic time-kill assays combined with mathematical modeling. Although MIC values remain the clinical standard, they cannot resolve the differences in growth and kill kinetics that emerge immediately after antibiotic addition. Recording full time-kill curves in different media conditions and fitting them with PD models revealed that identical MICs can mask substantial variation in growth and kill rates[48]. These lessons underline the need to complement standard AST with kinetic assays that explicitly incorporate nutrient context.

Both the nutrient and oxygen environments exert a significant influence on these growth and killing dynamics, as they are often associated with slow growth, tolerance, and persistence effects. In **Chapter 3**, a sigmoidal concentration–effect model was employed to more accurately describe the

antibiotic PD changes under varying nutrient conditions. Although this approach enabled the study of a wide range of antibiotic–nutrient combinations, it also presents clear disadvantages when moving towards more antibiotic-specific responses. The response data used to design our one-compartment PD model were obtained from a static time-kill assay, which fails to accurately capture biphasic killing curves of heterogeneous populations, exhibits variability depending on bacterial population size, and fails to track bacterial adaptation during antibiotic exposure. These limitations highlight the need for dynamic *in vitro* culturing systems, which could provide deeper insight into time-dependent PD characteristics like shifting growth and killing kinetics and resistance development[49].

Chapters 3 and 4 demonstrated that nutrient availability strongly influences antibiotic sensitivity, and in **Chapter 6** genetic outcomes of resistance evolution under these varied conditions were observed. To bridge these findings, PD models capturing the full time course of antibiotic effects can reveal differences in antibiotic selection pressure. These PD differences can subsequently inform population genetic models, linking observed antibiotic sensitivity shifts directly to genetic variation[50]. The complex interplay between nutrient conditions, bacterial growth rates, and antibiotic susceptibility highlights the difficulty of accurately replicating selective pressures *in vitro*. Although continuous culture systems (e.g., chemostats) are technically challenging to maintain, they offer more precise control. Chemostats allow microbial populations to be maintained in a constant environment over extended periods, while enabling deliberate manipulation of specific selective forces such as nutrient levels or antibiotic exposure[51].

Molecular drivers of nutrient induced antibiotic sensitivity changes

An important aspect highlighted throughout this thesis is that changes in antibiotic effect are often unique to specific nutrient-antibiotic combinations. While significant progress has been made through the rapid development of genomic databases cataloging resistance-conferring mutations ('resistomes')[52], these approaches primarily correlate known mutations with antimicrobial resistance profiles. Less conventional mechanisms, such as metabolism-driven resistance, are often underrepresented in these databases, as they are embedded within more complex networks[25]. Resistance-associated mutations in such

networks typically arise after prolonged periods of selection pressure (**Chapter 6**), whereas phenotypic adaptive responses can cause a transient reduction in antibiotic efficacy during earlier phases of exposure[48], exemplified in **Chapter 3**. Although this thesis did not explicitly investigate molecular mechanisms, the observed nutrient-antibiotic-specific effects emphasize the need for future studies to dissect the specific biochemical responses involved.

Transcriptomics, proteomics, and metabolomics probe molecular layers that lie much closer to the phenotype than genomic data, and thus provide the biochemical resolution needed to dissect antibiotic-response mechanisms. For example, whole-genome sequencing of two *P. aeruginosa* isolates accounted for only part of their divergent β-lactam phenotypes, whereas transcriptomics uncovered additional resistance-linked expression changes invisible at the DNA level[53]. Likewise, quantitative proteomics detected the early induction of heat-shock chaperones, proteases, and metabolic enzymes when *P. aeruginosa* was exposed to sub-inhibitory tobramycin, highlighting rapid adaptive pathways[54]. In **Chapter 5**, it was shown how metabolomics can track nutrient utilization, antibiotic-induced metabolite signatures[21], and how those metabolic read-outs can be exploited by supplementing targeted nutrients to enhance antibiotic sensitivity[9]. Each technique carries its own analytical limitations and, most critically, captures only a snapshot of an inherently dynamic system.

Fluxomics adds this missing temporal dimension by tracing how carbon and energy flow through metabolic networks under changing conditions. Such data reveal compensatory pathways that support survival during nutrient stress and antibiotic pressure[55]. Yet, building isolate-specific flux models for CF infections is data-intensive, and the necessary parameters for making robust extrapolations are seldom available.

Recent genome-scale metabolic reconstructions, which integrate hundreds of reactions and multi-omics inputs, improve confidence in flux predictions. For example, a model combining transcriptomics, proteomics, and metabolomics successfully captured lipopolysaccharide remodeling during polymyxin resistance in *P. aeruginosa*[56]. These integrative models better capture time-dependent processes such as fluctuating drug concentrations or nutrient availability.

Most -omics platforms are advancing toward single-cell resolution because that level of detail can reveal subtle phenotypic adaptations during

antibiotic exposure. Yet, despite their analytical power, single-cell studies have limited biological impact if they are not anchored to a broader mechanistic framework. The heterogeneity of bacterial populations is well established, as repeatedly illustrated by phenomena like antibiotic persistence[57]. Instead, research efforts should begin with established analytical techniques, integrate their datasets, and first map *P. aeruginosa* responses to each antibiotic. Once the antibiotic mode of action and the population-level biochemical responses have been well defined, single-cell approaches can then illuminate the fine-scale heterogeneity that underlies treatment failure.

7.3. Overall conclusion

In conclusion, this thesis demonstrates that shifts in nutrient and oxygen availability can rewire *P. aeruginosa* physiology and alter antibiotic pharmacodynamics. These findings confirm that effective drug evaluation must consider antibiotic mechanism, pathogen physiology, and microenvironment as interdependent factors, especially for heterogeneous infection sites such as the CF lung. The work presented here lays the groundwork for explicitly incorporating nutrient and oxygen levels into antimicrobial sensitivity assay development and systems-level analyses of bacterial responses. Looking ahead, combining detailed infection-site mapping with environment-aware experimental assays represents a necessary first step toward patient-specific antibiotic therapy.

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How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 8

Nederlandse samenvatting

8.1. Algemene introductie

Pseudomonas aeruginosa: een hardnekkige ziekteverwekker

Pseudomonas aeruginosa is een bacterie die goed gedijt op vochtige plekken, zoals stilstaand water, beekjes of vochtige aarde. Hoewel deze bacterie op veel plaatsen voorkomt, is deze bacteriesoort voor de meeste mensen geen ziekteverwekker. Voor mensen met een verzwakt afweersysteem kan *P. aeruginosa* echter wel gevaarlijk zijn: hun afweer is vaak niet sterk genoeg om de bacterie onder controle te houden. Omdat *P. aeruginosa* alleen onder bepaalde omstandigheden ziekte veroorzaakt, wordt de bacteriesoort beschouwd als een **opportunistisch pathogeen**. Maar omdat de bacterie niet alleen voorkomt in vochtige plekken in de natuur, maar ook in waterleidingen, kranen en airconditioningsystemen, kan ze zich gemakkelijk verspreiden in ziekenhuizen en verzorgingshuizen. Precies de plekken waar veel zieke en kwetsbare mensen verblijven. Hierdoor is *P. aeruginosa* verantwoordelijk voor een aanzienlijk deel van de infecties die patiënten tijdens hun ziekenhuisopname oplopen. Voorbeelden hiervan zijn urineweginfecties via katheters, bloedbaaninfecties bij infusen, of longontstekingen bij beademde patiënten. Wanneer iemand zo'n infectie oploopt, wordt vaak gestart met een **antibioticakuur**: een behandeling die gericht is op het doden van de bacterie. Hoewel deze behandeling in veel gevallen effectief is, komt het steeds vaker voor dat de infectie niet verdwijnt omdat het pathogeen niet meer gevoelig is voor de antibiotica (**Box 1**).

Box 1. De effectiviteit van antibiotica bij infecties

Het eerste **antibioticum** werd bij toeval ontdekt in 1928. Een schimmel scheidde een stofje uit dat de groei van omliggende bacteriën remde. Pas in 1940 slaagden wetenschappers erin om dit middel, penicilline, op grote schaal te produceren. Antibioticabehandelingen waren namelijk essentieel om infecties als gevolg van verwondingen of operaties te voorkomen, juist in een periode waarin de druk op het medisch personeel enorm was, tijdens de Tweede Wereldoorlog. Sindsdien is antibiotica niet meer weg te denken uit de gezondheidszorg. Dankzij deze middelen kunnen jonge kinderen die vroeger dagenlang ziek op de bank lagen, soms al na één dag behandeling weer vrolijk rondlopen. Ook ingrijpende operaties zijn een stuk veiliger geworden, omdat het risico op levensbedreigende infecties achteraf sterk is afgenomen.

Maar tegelijkertijd met de doorbraak van antibiotica werden ook de eerste bacteriën waargenomen die niet meer geremd werden bij blootstelling aan deze middelen. Deze

bacteriën ontwikkelden **antibioticaresistentie** door de doelwitten van antibiotica zodanig aan te passen dat de werking ervan verloren ging. Omdat bacteriën in grote populaties voorkomen en zich zeer snel delen, kan een verandering die een overlevingsvoordeel biedt snel dominant worden binnen een populatie. Deze snelle **evolutionaire aanpassing** leidde ertoe dat al kort na de introductie van antibiotica bacterielijnen ontstonden die volledig resistent waren, zoals de bekende ziekenhuisbacterie methicilline-resistente *Staphylococcus aureus* (MRSA).

Om infecties met antibioticaresistente bacteriën toch te kunnen blijven behandelen, zijn er in de loop der tijd veel nieuwe antibiotica ontwikkeld. Dit heeft geleid tot een breed scala aan **antibioticaklassen** met verschillende werkingsmechanismen. Om de werking van antibioticaklassen te waarborgen gelden er strikte voorschrijfregels. Toch overlijden jaarlijks nog altijd miljoenen mensen aan infecties waarbij antibioticaresistente bacteriën een rol spelen. Vaak omdat deze bacteriën ongevoelig zijn voor meerdere antibioticaklassen.

Hoewel onverantwoord gebruik van antibiotica vaak wordt genoemd als belangrijke aanjager van de ontwikkeling van **multiresistente bacteriën**, valt dit buiten de reikwijdte van dit proefschrift. Dit proefschrift richt zich op infecties die klinisch moeilijk te behandelen zijn doordat bacteriën zich tijdens de infectieperiode kunnen aanpassen door falende antibiotica therapieën. Als gevolg hiervan kunnen infecties langdurig blijven bestaan (soms maanden tot zelfs jaren), en dus overgaan in een **chronisch** ziektebeeld.

P. aeruginosa infecties in de longen van cystische fibrosepatiënten

Cystische fibrose (CF), ook wel taaislijmziekte genoemd, is een genetische aandoening die leidt tot een abnormaal dikke slijmlaag in de luchtwegen. In gezonde longen helpt het slijm bij het afvoeren van pathogenen en afvalstoffen. Bij CF-patiënten is het slijm gedehydrateerd en stroperig, waardoor deze klaringsfunctie ernstig verstoord is. Dit creëert een gunstige omgeving voor micro-organismen om zich in de longen te vestigen. Het afweersysteem reageert op deze kolonisatie, wat leidt tot ontstekingsreacties en infecties. Al op jonge leeftijd worden bij CF-patiënten herhaaldelijk bacteriële kolonies aangetroffen in de luchtwegen. Om deze kolonies onder controle te houden en verdere longschade te beperken, zijn CF-patiënten vaak aangewezen op langdurige onderdrukkende antibioticatherapieën. Helaas zijn deze behandelingen bij een aanzienlijk deel van de patiënten niet effectief genoeg om chronische longinfecties volledig te voorkomen. In sommige gevallen zijn de infecties zo

hardnekkig en therapieresistent dat een risicovolle longtransplantatie de enige overgebleven optie is om de infectie te elimineren.

P. aeruginosa is één van de meerdere pathogenen die verantwoordelijk zijn voor chronische longinfecties bij CF-patiënten. Wat *P. aeruginosa* specifiek maakt, is dat de infectie vaak al op jonge leeftijd wordt opgelopen en vervolgens niet meer volledig geëlimineerd kan worden. Hierdoor is *P. aeruginosa* de meest dominante pathogeen in de luchtwegen van volwassen CF-patiënten. Meer inzicht in de manier waarop de chronische *P. aeruginosa* infectie ontstaat en de respons op antibiotica bij CF-patiënten biedt niet alleen kansen om de klinische uitkomst voor deze specifieke patiëntengroep te verbeteren, maar is ook van groot belang om het groeiende probleem van infecties met multiresistente *P. aeruginosa*-stammen het hoofd te bieden.

De stofwisseling van *P. aeruginosa* in de longen van CF-patiënten

Door de vorming van een dikke laag slijm en het gebrek aan effectieve klaring ontstaat een unieke leefomgeving in de **mucus** van de luchtwegen van CF-patiënten. In deze directe, lokale **micro-omgeving** waarin *P. aeruginosa* zich vestigt, hopen potentiële voedingsstoffen zich op, terwijl de hoeveelheid beschikbare zuurstof zeer beperkt is. Door de slechte zuurstofpenetratie in de mucus ontstaan zuurstofarme tot zelfs volledig zuurstofvrije omstandigheden. De stofwisseling van *P. aeruginosa* speelt een belangrijke factor waarom de bacterie zich gemakkelijk kan vestigen in deze unieke micro-omgevingen.

Het veelzijdige en flexibele metabolisme van *P. aeruginosa* sluit goed aan op het aanbod voedingsstoffen in de mucus van de longen van CF-patiënten (**Box 2**). In de mucus worden namelijk hoge concentraties gemeten van de **nutriënten** die *P. aeruginosa* efficiënt benut: aminozuren, korte-keten organische zuren, en nitraat. Daarnaast kan *P. aeruginosa* grotere moleculen actief afbreken om de gewenste nutriënten vrij te maken, en beschikt het over **virulentiefactoren** die het vrijkomen van nutriënten in humane cellen forceren. Dankzij deze overvloed aan beschikbare nutriënten kan de bacterie zich gemakkelijk vestigen in zowel zuurstofrijke (**aerobe**) als zuurstofarme (**anaerobe**) micro-omgevingen in de longen van CF-patiënten.

Box 2. Metabolisme van *P. aeruginosa*

Het **metabolisme** van *P. aeruginosa* wijkt af van veel andere pathogenen en humane cellen. In humane cellen start de energieproductie meestal met suikers zoals **glucose**, die worden afgebroken tot kleinere moleculen. Deze tussenproducten kunnen dienen als bouwstenen, of in combinatie met zuurstof worden gebruikt in **oxidatieve fosforylatie**. Tijdens oxidatieve fosforylatie wordt de energie uit de suikerafbraak opgeslagen in de vorm van **ATP**. Het ATP-molecuul is de universele energiedrager van cellen. In humane cellen vindt de oxidatieve fosforylatie plaats in de mitochondriën, die daardoor de bijnaam 'energiecentrales van de cel' dragen. Hoewel oxidatieve fosforylatie een zeer efficiënte manier is van energieproductie, vereist het de aanwezigheid van zowel suikers en zuurstof. *P. aeruginosa* daarentegen maakt juist veelvuldig gebruik van alternatieve koolstofbronnen, zoals **aminozuren** en korte-keten **organische zuren**. Deze aminozuren en organische zuren zijn kleine moleculen die fungeren in humane cellen ook als belangrijke bouwstenen voor macromoleculen zoals eiwitten en lipiden. Maar *P. aeruginosa* beschikt over meerdere alternatieve metabole routes om deze kleine moleculen efficiënt te benutten voor energieproductie. Het gebruik van deze alternatieve nutriënten geven *P. aeruginosa* niet alleen een voordeel in de concurrentie om nutriënten, maar zorgt ook voor een hoge mate van flexibiliteit.

De metabole flexibiliteit van *P. aeruginosa* wordt verder vergroot doordat de bacterie, naast zuurstof, ook **nitraat** kan gebruiken als alternatief voor het proces om energie op te slaan in de vorm van ATP. Dit proces, **denitrificatie** genoemd, stelt *P. aeruginosa* in staat om ook in anaerobe omgevingen waar geen zuurstof aanwezig is efficiënt energie te blijven produceren. Wanneer zowel zuurstofrespiratie als denitrificatie niet mogelijk zijn, kan *P. aeruginosa* terugvallen op **fermentatie** en **deiminatie**. Fermentatie is een anaerobe metabole route voor energieproductie, vergelijkbaar met wat er in spieren gebeurt tijdens zware inspanning: glucose wordt dan deels afgebroken zonder dat zuurstof nodig is, met **lactaat** productie als gevolg. Bij deiminatie worden aminozuren afgebroken via specifieke enzymatische routes waarbij eveneens ATP wordt gegenereerd. Deze metabole veerkracht stelt *P. aeruginosa* in staat zich uitstekend aan te passen aan de vaak anaerobe mucus in de longen van CF-patiënten.

Het metabolisme van bacteriën beïnvloed de efficiëntie van antibiotica

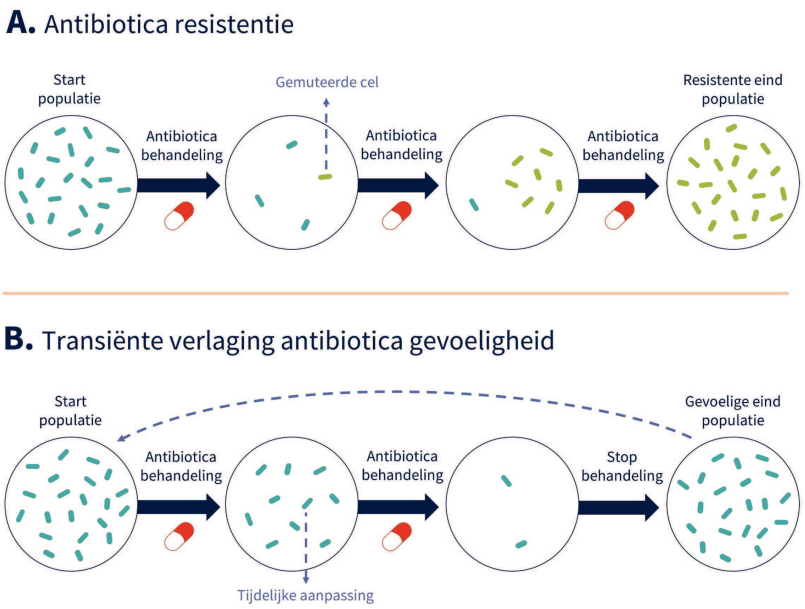
Bacteriën beschikken over meerdere mechanismen om de werking van antibiotica te verminderen of zelfs volledig uit te schakelen. Antibiotica resistentie is verantwoordelijk voor het overgrote deel van de miljoenen sterfgevallen die jaarlijks wereldwijd worden toegeschreven aan het falen van

antibiotica behandelingen. Er is dan ook veel onderzoek gedaan naar de mechanismen die leiden tot antibioticaresistentie.

Door antibiotica **resistentiemechanismen** is een bacterie in staat zijn om te blijven delen bij antibiotica-concentraties die normaal gesproken de groei van de populatie remmen of dodelijk zijn (**Figuur 1A**). Veelvoorkomende resistentiemechanismen zijn: (1) enzymatische afbraak of modificatie van het antibioticum, (2) actieve efflux, waarbij antibiotica uit de cel worden gepompt, (3) veranderingen in het doelwit van het antibioticum, waardoor het niet meer effectief kan binden, en (4) verminderde opname of doorlaatbaarheid van het antibioticum.

Deze resistentiemechanismen ontstaan door veranderingen in het bacteriële DNA. Een voorbeeld hiervan is een spontane mutatie in het stukje DNA dat de informatie bevat voor het maken van de eiwitten waaraan een antibioticum normaal bindt om celprocessen te verstoren. Als de aanpassing in het DNA ervoor zorgt dat het antibioticum niet meer goed kan binden, wordt deze specifieke bacteriecel ongevoelig voor het antibioticum. Omdat DNA wordt doorgegeven aan nakomelingen, zullen ook de nieuwgevormde cellen dezelfde aanpassing in het DNA hebben. De bacteriecellen die de mutatie niet hebben en dus nog wel gevoelig zijn voor het antibioticum, zullen tijdens de behandeling afsterven of stoppen met delen. Daardoor kunnen de resistente cellen met de mutatie snel de overhand nemen binnen de bacteriepopulatie. De kans op het ontstaan van zo'n mutatie die deze **evolutie** in gang zet is op zichzelf klein. Maar omdat een infectie vaak uit miljoenen bacteriën bestaat die zich bovendien zeer snel kunnen verdubbelen, is de kans dat er ergens in de populatie een resistente variant ontstaat reëel. Zeker bij de langdurige antibioticatherapieën die gebruikt worden om longschade bij CF-patiënten te beperken.

Naast deze stabiele, genetische resistentiemechanismen maken bacteriën ook gebruik van tijdelijke strategieën waarmee ze een antibiotica behandeling tijdelijk kunnen overleven. Een infectie met een pathogeen dat zulke **transiënte mechanisme** toepast lijkt tijdens de behandeling vaak succesvol bestreden, maar keert terug zodra de antibiotica wordt stopgezet (**Figuur 1B**). Bij hervatting van dezelfde behandeling is de bacterie meestal opnieuw gevoelig, omdat het gaat om



Figuur 1. Visualisatie van hoe bacteriële populaties reageren op antibiotische behandeling. (A) antibiotica resistentie: een stabiele genetische verandering zoals een mutatie zorgt ervoor dat bacteriën kunnen blijven groeien tijdens blootstelling aan antibiotica. (B) Transiënte verlaging van antibiotica gevoeligheid: door een fenotypische verandering ontstaat een verlaagde gevoeligheid, waardoor bacteriën de antibiotische werking tijdelijk overleven.

een tijdelijke **fenotypische** aanpassing. Er vindt dus geen stabiele verandering plaats in het DNA. Toch vergroten deze tijdelijke aanpassingen de kans dat op termijn wel stabiele, genetische resistentie ontstaat en zich vastzet binnen de bacteriële populatie.

Het bacteriële metabolisme speelt een belangrijke rol in zulke tijdelijke veranderingen in antibioticagevoeligheid. Dit is duidelijk zichtbaar bij de werking van **aminoglycoside**-antibiotica. Aminoglycosiden, zoals **tobramycine**, verstoren de eiwitsynthese in bacteriën, wat uiteindelijk leidt tot celdood. De opname van deze antibiotica in de bacteriecel is echter afhankelijk van een elektrisch spanningsverschil over het celmembraan, dat wordt opgewekt tijdens de energieproductie via oxidatieve fosforylatie. Hierdoor hopen aminoglycosiden zich vooral op in metabool actieve cellen met een hoog energieniveau, en is de

aminoglycoside effectiever in celdoding. Dit staat in contrast met de werking van **polymyxines**, een andere klasse van bacteriedodende antibiotica. Polymyxines, zoals **colistine**, binden aan lipiden in de buitenste laag van de celwand. Om deze binding te verhinderen, kan de bacterie de samenstelling van de lipiden in de celwand actief aanpassen. Echter kost dit proces veel energie en dus niet mogelijk is in cellen met een laag energieniveau. Daardoor zijn polymyxines juist dodelijker voor cellen met een lage metabole activiteit.

Net als bij mensen verschillen bacteriecellen binnen een populatie van elkaar, zowel in genotypisch als fenotypisch opzicht. Deze **heterogeniteit** tussen cellen maakt dat een bacteriepopulatie zich goed kan aanpassen aan veranderende omstandigheden. Een goed voorbeeld van variatie tussen *P. aeruginosa* cellen in de luchtwegen van CF-patiënten zijn **biofilm-aggregaten**. Biofilms bestaan uit bacteriën die hun beweeglijkheid opgeven en samen een beschermende matrix uitscheiden. Deze extracellulaire matrix beschermt de bacteriën tegen schadelijke omgevingsfactoren, zoals antibiotica en het afweersysteem. Naast deze bescherming zorgt de matrix er ook voor dat voedingsstoffen beperkt tot de kern van de biofilm komen. Daardoor ontstaat in de kern een voedselarme en zuurstofarme micro-omgeving, waarin bacteriën inactief of **dormant** raken. Colistine werkt vooral goed op de inactieve cellen diep in de biofilm, terwijl tobramycine juist effectief is tegen de actieve bacteriën aan de buitenkant. Het toevoegen van voedingsstoffen tijdens de antibiotica behandeling zorgt dat bacteriën actiever worden, waardoor onder andere tobramycine beter gaat werken. Dit benadrukt hoe belangrijk het is om bij de keuze van een behandeling niet alleen naar het antibioticum te kijken, maar ook naar de metabole activiteit van de bacterie.

De micro-omgevingen beïnvloeden *P. aeruginosa* antibiotica gevoeligheid

De werking van antibiotica hangt nauw samen met de metabole activiteit van *P. aeruginosa*, die grotendeels wordt bepaald door de hoeveelheid beschikbare nutriënten en zuurstof in de omgeving. In de longen van CF-patiënten bestaan veel verschillende micro-omgevingen, die ontstaan door variatie in de ernst van de ziekte, de snelheid van longfunctieverlies, bijkomende infecties en de structuur van de luchtwegen. *P. aeruginosa* past zijn metabolisme aan deze omstandigheden aan, wat leidt tot een breed scala aan resistentiemechanismen.

Het is daarom belangrijk om te begrijpen welke specifieke veranderingen in de omgeving invloed hebben op de antibioticagevoeligheid van *P. aeruginosa*. Dit inzicht is essentieel om terugkerende behandelproblemen te voorkomen en chronische infecties beter onder controle te krijgen.

Analyse van bacteriële groei en antibioticawerking in het laboratorium

Om het effect van de groeicondities op antibioticagevoeligheid te bestuderen, zijn gecontroleerde omstandigheden nodig. In het menselijk lichaam wordt de omgeving beïnvloed door talloze factoren, waardoor het lastig is om het effect van één specifieke factor te isoleren. Daarom wordt vaak uitgeweken naar ***in vitro*** laboratoriumonderzoek.

In de kliniek wordt bijvoorbeeld het pathogeen uit een patiënt geïsoleerd om de gevoeligheid voor antibiotica te bepalen. In het laboratorium worden deze bacteriën vervolgens gekweekt onder gecontroleerde omstandigheden: bij lichaamstemperatuur (37 °C) en in een voedingsrijk **kweekmedium**. Vervolgens worden de bacteriën blootgesteld aan een verdunningsreeks van een antibioticum, waarbij elke volgende concentratie het dubbele is van de vorige. Na 20 tot 24 uur incubatie wordt het effect beoordeeld door te kijken naar de troebelheid van het medium: als het medium troebel is geworden, betekent dit dat de bacterie groeit ondanks de aanwezigheid van het antibioticum. De laagste concentratie waarbij geen zichtbare groei optreedt, wordt de **minimale inhibitieconcentratie (MIC)** genoemd.

Hoewel de MIC een belangrijke maat is voor de gevoeligheid van een bacterie voor een antibioticum, heeft deze methode ook een aantal beperkingen. Zo geeft de MIC slechts een momentopname weer, en wordt de groei of afname van de bacterie in de tijd niet in kaart gebracht. Daarnaast worden gestandaardiseerde kweekmedia gebruikt die de complexe omgeving van de longen in CF-patiënten niet goed nabootsen, waardoor de resultaten niet altijd representatief zijn voor de situatie in het lichaam.

Om de beperking van de meting op één enkel tijdspunt bij MIC-bepalingen te omzeilen, kunnen **time-kill** experimenten worden gebruikt. In deze

Box 3. Bepalen van de bacteriedichtheid

De bacteriedichtheid kan op verschillende manieren worden bepaald. De snelste en goedkoopste methode is het meten van de optische dichtheid (OD). Dit is vergelijkbaar met de eindmeting van een MIC-test, maar kan bij time-kill experimenten dynamisch worden uitgevoerd met behulp van een fotospectrometer. De fotospectrometer schijnt licht met een golflengte van 600 nanometer door het kweekmedium. Dit licht wordt deels geabsorbeerd en verstrooid, waardoor er minder licht de detector bereikt. De gemeten waarde, **OD_{600}**, geeft een indicatie van de troebelheid van het medium. Omdat bacteriën de dichtheid van het medium verhogen, neemt de OD_{600}-waarde toe met de groei van de cultuur. De OD_{600} zegt echter niets over het aantal levende bacteriën, omdat ook dode cellen het licht tegenhouden. Hierdoor is OD_{600} niet geschikt om celdoding door antibiotica vast te stellen. Om zowel groei als afname van levende bacteriën te meten, wordt daarom vaak gebruikgemaakt van het tellen van **colony forming units (CFU)**. Eén CFU staat voor een levende bacterie die in staat is een kolonie te vormen op een voedingsbodem in een petrischaal. Aangezien bacterieculturen vaak uit tientallen miljoenen bacteriën bestaan, wordt de cultuur verdund om het aantal kolonies telbaar te maken. Het maken van verdunningen, incuberen van de platen en handmatig tellen van CFU's is echter tijdrovend. Daarom kan een indirecte bepaling van levende cellen veel tijd besparen. In dit proefschrift is hiervoor gebruikgemaakt van **bioluminescente** bacteriën. Deze bacteriën zijn genetisch aangepast om een eiwit te produceren dat voortdurend licht uitzendt. Dit eiwit functioneert alleen in levende cellen, omdat het ATP nodig heeft om luminescentie te genereren. Wanneer bacteriën afsterven, daalt het ATP-gehalte en neemt het uitgezonden licht af. Dankzij deze methode kan de bacteriële dichtheid gedurende het time-kill experiment volledig automatisch worden gevolgd met een fotospectrometer.

experimenten wordt de bacteriedichtheid gedurende de tijd gevolgd (**Box 3**), waardoor het mogelijk is om het groeiverloop en de mate van afname door antibiotica nauwkeurig te bepalen. In tegenstelling tot de statische MIC-meting kunnen time-kill experimenten subtiele veranderingen in groeidynamiek zichtbaar maken, zoals tijdelijke overlevingsstrategieën of het opnieuw opkomen van groei door het ontstaan van resistentie (**Hoofdstuk 1, Figuur 1**).

De bacteriedichtheid over tijd uit het time-kill experiment kan vervolgens worden gebruikt als input voor **farmacodynamische (PD) modellen**. Deze wiskundige modellen beschrijven de relatie tussen concentratie van het antibioticum en het effect op de bacteriedichtheid (**Box 4**). De parameters uit het model geven daarmee een kwantitatieve beschrijving van de **concentratie-effectrelatie** van

het antibioticum en de bacterie. Op deze manier kunnen ook subtiele veranderingen in groeidynamiek uit het time-kill experiment worden meegenomen bij het vergelijken van antibiotica of groeicondities.

Box 4. Farmacometrie

Farmacometrie is het vakgebied dat focust op het kwantitatief modelleren en analyseren van de werking van geneesmiddelen in het lichaam. Het combineert farmacokinetiek en farmacodynamiek met statistiek en wiskunde om te begrijpen, voorstellen en optimaliseren hoe medicijnen werken bij patiënten. In antibioticastudies wordt farmacometrie gebruikt om de juiste dosis en toedieningsfrequentie te bepalen die nodig zijn om een zo effectief mogelijk effect op de bacteriën te bereiken.

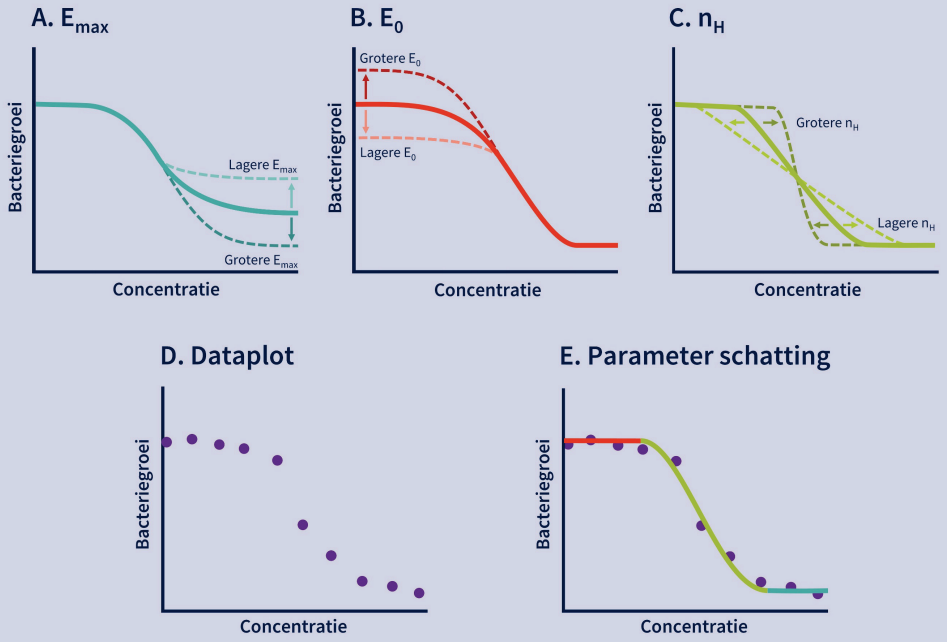
Farmacokinetiek (PK) beschrijft hoe het lichaam invloed uitoefent op de concentratie van een geneesmiddel. Denk hierbij aan processen zoals de opname in de darmen, de verdeling binnen het lichaam, de afbraak van het middel en de uitscheiding ervan. In een PK-model worden deze processen wiskundig weergegeven met parameters die de concentratie beïnvloeden. Op basis hiervan kan het verloop van de geneesmiddelconcentratie tijdens een behandeling worden gesimuleerd en weergegeven in een concentratie-tijd grafiek.

Farmacodynamiek (PD) beschrijft het effect van het geneesmiddel op het lichaam of micro-organismen. In dit proefschrift is het effect de afname van bacteriegroei. Door meerdere time-kill experimenten uit te voeren met verschillende concentraties kan een verschil in effect bepaald worden. De concentratie-effect relatie wordt vervolgens met een wiskundig model beschreven, zodat ook het effect bij concentraties die niet zijn getest gesimuleerd kunnen worden. In dit proefschrift wordt een E_{max} model gebruikt om de concentratie-effect relatie te beschrijven (**vergelijking 1**).

$$Bacteriegroei([AB]) = E_{max} + \frac{E_0 - E_{max}}{1 + e^{n_H(\log([AB]) - \log(EC_{50}))}} \quad (1)$$

Een E_{max} model beschrijft hoe sterk het effect is van een antibioticum door de relatie te bepalen tussen 2 asymptoten. Het maximale effect van het antibioticum wordt beschreven in de onderste asymptoot, de E_{max} (**Figuur 2A**). De concentraties waarbij nog geen effect waarneembaar is wordt beschreven door de bovenste asymptoot, de E_0 (**Figuur 2B**). En het

effect-concentratie tussen de asymptoten door de Hill coëfficiënt, de n_H (**Figuur 2C**). De computer berekent de modelparameters die het beste passen bij de metingen uit het time-kill experiment, door de fout tussen model en data zo klein mogelijk te maken (**Figuur 2D-E**).



Figuur 2. Het beschrijven van de concentratie-effect relatie met het E_{max} model. De figuren A-E zijn een concentratie-effect plot met op de Y-as het effect aangegeven met bacteriegroei en op de x-as de concentratie van het antibioticum. Bij een hogere concentratie van het antibioticum zal de bacteriegroei verminderen, waardoor het effect groter is.

Om tijdens deze laboratorium **in vitro** experimenten beter de omstandigheden in het lichaam na te bootsen, is in 2007 een **synthetisch CF-sputum medium (SCFM)** ontwikkeld. Dit kweekmedium lijkt qua nutriënten op het mucus van CF-patiënten. *P. aeruginosa* gedraagt zich in SCFM gelijkwaardiger aan hoe ze zich in het lichaam gedragen dan wanneer ze in standaard laboratorium media worden gekweekt. Omdat *P. aeruginosa* anders gedraagt in de verschillende media, reageren ze ook anders op antibiotica. Hoewel SCFM dus beter lijkt op de echte situatie in de longen, geeft een MIC-test met slechts één soort kweekmedium nog steeds geen volledig beeld. In de longen van CF-patiënten zijn namelijk veel verschillende soorten voedingsomgevingen aanwezig.

Daarnaast kan de invloed van voeding op het gedrag van de bacterie en de reactie op antibiotica beter worden onderzocht met behulp van PD modellen (**Hoofdstuk 1, Figuur 2**). De veranderingen in antibiotica gevoeligheid worden vaak grof geclassificeerd op basis van de PD veranderingen. Zo wordt bijvoorbeeld groei tijdens antibiotica behandeling geclassificeerd als resistentie, terwijl veranderingen in de dynamiek van bacteriedoding vaak worden toebedeeld aan transiënte mechanisme. Maar deze veranderingen in antibiotica PD zijn het gevolg van ingewikkelde veranderingen in onderliggende cel-processen die zich uiten in de fenotypische veranderingen. Omdat de voedingsomgeving ook effect heeft op dezelfde cel-processen, speelt dit ook een rol op het fenotype van de bacterie in het vormen de PD uitkomsten.

De veranderingen die optreden in bacteriën tijdens een antibioticabehandeling variëren van aanpassingen in het DNA tot veranderingen in de stofwisseling. Het bestuderen van deze processen is belangrijk om beter te begrijpen waarom antibiotica soms wel en soms niet goed werken. Uiteindelijk helpt dit om PD onderzoeksresultaten beter te vertalen naar effectieve behandelstrategieën in de kliniek. Dit onderzoek gaat enerzijds om het genetisch in kaart brengen van mutaties in bacteriën die de gevoeligheid voor antibiotica beïnvloeden. Maar ook om het toepassen van andere omics-technieken zoals transcriptomics, proteomics en **metabolomics** om fenotypische veranderingen in bacteriën te volgen (**Box 5**). Vooral bij transiënte veranderingen in antibioticagevoeligheid zijn deze technieken waardevol, bijvoorbeeld als reactie op verschillen in nutriënten. Door de resultaten van deze omics-methodes te combineren, ontstaat een beter beeld van hoe de omgeving en de bijbehorende bacteriële aanpassingen de werking van antibiotica beïnvloeden op moleculair niveau. Dit inzicht maakt het mogelijk om laboratoriummodellen op specifieke onderdelen realistischer te maken, zodat bacteriën onder testomstandigheden meer lijken op hoe ze zich gedragen in het lichaam van de patiënt. Daardoor kunnen behandelingen beter afgestemd worden op specifieke ziektebeelden. Daarnaast is het ontwikkelen van realistische modellen belangrijk voor het testen van nieuwe antibiotica. Of een nieuw middel kans maakt op verdere klinische ontwikkeling, hangt sterk af van hoe goed het werkt in laboratoriumtests. Een testomgeving die beter aansluit bij de situatie in het lichaam bij moeilijk te behandelen infecties verhoogt de voorspellende waarde van zulke experimenten.

Voor luchtweginfecties bij CF-patiënten is de kennis voor het verbeteren van laboratoriummodellen ook belangrijk. De longomgeving bij CF wordt gekenmerkt door een unieke samenstelling van nutriënten, terwijl *P. aeruginosa* het vermogen om zich flexibel fenotypisch aan te passen. Deze complexe wisselwerking vraagt om een gestructureerde en stapsgewijze benadering. Die begint bij het systematisch onderzoeken van afzonderlijke omgevingsfactoren. Daarmee wordt de basis gelegd voor beter inzicht in hoe de CF-longomgeving, het bacteriële metabolisme en antibiotica elkaar beïnvloeden.

Box 5. -Omics benadering

"Meten is weten" is het uitgangspunt van de -omics-technieken. Het woord -omics verwijst naar een groep onderzoeksvelden die zich richten op het in kaart brengen van verschillende lagen binnen een cel. Elk van deze velden bestudeert een ander aspect van hoe een cel werkt. De naam van deze onderzoeksgebieden eindigt op -omics, zoals genomics, transcriptomics, proteomics, en metabolomics (**Tabel 1**). Door deze lagen systematisch te meten, kunnen onderzoekers beter begrijpen hoe een cel zich aanpast aan zijn omgeving, bijvoorbeeld bij stress of blootstelling aan antibiotica.

Tabel 1. Voorbeelden van -omics velden.

Veld	Wat het meet	Toepassing
Genomics	Het volledige DNA	Identificeert genetische veranderingen, zoals mutaties of resistentiegenen
Transcriptomics	De RNA-moleculen die worden afgelezen van het DNA	Laat zien welke genen op een bepaald moment actief zijn
Proteomics	De eiwitten die in de cel worden geproduceerd	Toont welke celprocessen en functies actief zijn
Metabolomics	Kleine moleculen (metabolieten) die ontstaan uit de stofwisseling	Geeft inzicht in de metabole activiteit van de cel

8.2. Doel van dit proefschrift

De impact van de fenotypische flexibiliteit van *P. aeruginosa* en de uiteenlopende micro-omgevingen in de CF-longen op de werking van antibiotica en het ontstaan van resistentie is nog onvoldoende begrepen. Om de behandelstrategieën met antibiotica voor chronische luchtweginfecties door *P. aeruginosa* bij CF-patiënten te verbeteren, is het essentieel om beter te begrijpen hoe de infectieuze micro-omgeving de respons op antibiotica vormgeeft. In dit proefschrift stellen wij de hypothese dat nutriëntcondities een centrale rol spelen in veranderingen in antibiotica PD en de evolutie van antibioticaresistentie beïnvloeden. Hierdoor hebben de nutriëntcondities uiteindelijk invloed op het therapeutisch resultaat.

8.3. Aanpassingen aan de verschillende omgevingen in de CF-long

Om onze huidige kennis met betrekking tot de hypothese van dit proefschrift samen te vatten, biedt **Hoofdstuk 2** (Sectie I) een uitgebreid overzicht van de metabole aanpassingen van *P. aeruginosa* aan de uiteenlopende nutriënt-niches in de longen van CF-patiënten. Deze metabole aanpassingen van de bacterie bepaalt onder meer de groeisnelheid, energiehuishouding, en hoeveelheid stress in de cellen. Deze factoren hebben allemaal invloed op de gevoeligheid voor antibiotica. In dit kader spelen de diverse micro-omgevingen met verschillende nutriënten en verschillen in zuurstofconcentraties een centrale rol in de werking van antibiotica behandelingen. Deze verschillen in micro-omgevingen worden nog verder versterkt binnen de voedingsstofgradiënten binnen biofilm-aggregaten van *P. aeruginosa*. Door deze verschillen in micro-omgevingen ontstaat er een metabole heterogeniteit binnen de infectie.

Om te begrijpen wat het effect is van de metabole heterogeniteit tussen *P. aeruginosa* cellen op antibiotica gevoeligheid is het belangrijk om de metabole flexibiliteit te begrijpen. *P. aeruginosa* is in staat om uiteenlopende substraten te benutten, zoals aminozuren en vetzuren, en kan moeiteloos schakelen tussen aerobe respiratie, fermentatie en denitrificatie om de energieproductie op peil te houden onder wisselende omstandigheden. Deze metabole plasticiteit beïnvloedt de gevoeligheid voor verschillende klassen van

antibiotica, waaronder aminoglycosiden, fluoroquinolonen, β-lactams en polymyxines. Deze antibioticaklassen zijn elk op een manier afhankelijk van specifieke metabolische toestanden om effectief te zijn. Naast dat *P. aeruginosa* het flexibele metabolisme gebruikt voor het aanpassen verschillende omstandigheden, kan het metabolisme ook actief herstructureerd worden om de effectiviteit van antibiotica vermindert.

Het begrijpen van deze metabole responsen in fysiologisch relevante contexten is cruciaal voor het ontwikkelen van effectievere en meer gepersonaliseerde antibioticabehandelingen. Hiervoor moet wel benoemd worden dat er binnen een patiënt (intra-) als tussen patiënten (inter-) aanzienlijke variatie is in de micro-omgevingen. Factoren zoals constructie van de longen, ziektebeelden die samengaan met CF, en het CF-genotype zorgen voor substantiële verschillen in nutriëntbeschikbaarheid, en dus in antibioticagevoeligheid. Daarom vereist vooruitgang het integreren van klinische inzichten met geavanceerde *in vitro*-modellen en multi-omics benaderingen die de complexiteit van de CF-long realistisch benaderen.

Op basis van de huidige stand van kennis en het benoemen van de hiaten beschreven in dit hoofdstuk is het onderzoek in dit proefschrift opgebouwd. Het onderzoek is vervolgens verdeeld in het effect van nutriënten op antibioticagevoeligheid (Sectie II) en als sturende factor in antibiotica resistentie-evolutie (Sectie III).

8.4. Voedingsstoffen beïnvloeden het effect van antibiotica

Nutriënten en zuurstof behoren tot de meest voor de hand liggende aanjagers van metabole processen, en deze processen worden in toenemende mate in verband gebracht met de werking van verschillende klassen antibiotica. Toch is de regulerende rol van specifieke omstandigheden die kenmerkend zijn voor de CF-long nog onvoldoende begrepen.

In **Hoofdstuk 3** is onderzocht hoe specifieke nutriënten de antibioticagevoeligheid van *P. aeruginosa* beïnvloeden door in een basaal basis

medium telkens één nutriënt toe te voegen. Door time-kill experimenten met een bioluminescente stam van *P. aeruginosa* uit te voeren hebben we populatie dynamiek onder veel omstandigheden kunnen analyseren.

Met behulp van PD modellering werden nutriënt-afhankelijke, antibioticaklasse-specifieke verschuivingen aangetoond. Deze patronen wijzen op onderliggende biochemische mechanismen en gaan verder dan de klassieke verklaring dat een lagere metabolische activiteit resulteert in lagere gevoeligheid. Daarnaast suggereert de afwezigheid van duidelijke verschillen in groeisnelheid tussen de verschillende nutriëntcondities dat de gevoeligheidsverandering niet primair door groeisnelheid wordt bepaald. Ook hebben *in vitro* PK-PD-simulaties lieten zien dat nutriënten de verwachte behandeluitkomst veranderd.

Hoewel de gebruikte *in vitro*-modellen beperkt zijn vergeleken met de complexiteit van klinische infecties, vormen deze resultaten een waardevolle stap vooruit. Ze vergroten ons begrip van de gepersonaliseerde aard van de behandeling van *P. aeruginosa*-infecties, die sterk worden beïnvloed door interacties met de lokale micro-omgeving.

Hoofdstuk 4 beschrijft hoe zuurstofbeschikbaarheid de antibioticagevoeligheid van *P. aeruginosa* beïnvloedt. In plaats van uitsluitend te focussen op acute reacties op anoxie, beoordeelde de studie ook hoe langdurige anoxische blootstelling (<1% zuurstof gedurende 22 dagen) de antibioticagevoeligheid beïnvloedt. Met time-kill experimenten werd deze anoxische geconditioneerde stam met de moederstam vergeleken. De effecten bleken antibioticum specifiek en de gevoeligheidsprofielen veranderden sterk na langdurige anoxie. Daarnaast liet de anoxische geconditioneerde stam ook zien dat er blijvende fysiologische aanpassingen waren onder normale zuurstofcondities. Dit onthult beperkingen van standaard *in vitro* experimenten en benadrukt de noodzaak voor kweekcondities die anoxische infectiecondities beter nabootsen. Hoewel het proefschrift vooral gericht op is CF, zijn deze inzichten waarschijnlijk ook relevant voor andere infectielocaties zoals in de urineweg, COPD-slijmproppen.

Samenvattend laat Sectie II zien dat metabole regulatie bepalend is voor de antibioticagevoeligheid van *P. aeruginosa* onder uiteenlopende nutriënt- en zuurstofcondities. Zelfs een enkele nutriëntverandering kan de gevoeligheid

substantieel verschuiven, afhankelijk van de combinatie nutriëntconditie-antibioticaklasse. Meer nutriënten betekent niet automatisch betere werking omdat de metabole flexibiliteit beschermende routes kunnen activeren die antibiotica tegenwerken. Door deze metabole regulatiesystemen zal het mixen van nutriënten in de geteste media niet per se additieve effecten opleveren. Dergelijke inzichten benadrukken het belang van het ontrafelen van de biochemische basis van nutriënt-gestuurde antibioticagevoeligheid en benadrukken de noodzaak van integratieve, fenotype-gerichte -omics benaderingen. Metabolomics en proteomics zijn krachtige technieken die subtiele verschuivingen in intracellulaire metabolietconcentraties, enzymniveaus kunnen vastleggen om metabole herconfiguraties tijdens antibioticabehandeling in kaart te brengen.

Voor deze biochemische benadering in toekomstig onderzoek zijn klinisch representatieve omgevingen essentieel. Naast de incrementele aanpak van het onderscheiden van de effecten van individuele nutriënten, is gedetailleerd onderzoek naar de nutriëntsamenstelling van de infectieuze omgeving essentieel. Daarbij moeten ook de interacties met andere cellen die het bacteriële metabolisme moduleren meegenomen worden. Door voort te bouwen op deze experimentele data kan toekomstig onderzoek nauwkeuriger rekening houden met de complexe nutritionele en metabole landschappen die *P. aeruginosa* benut om de antibioticagevoeligheid te beïnvloeden.

8.5. Nutriënten sturen de evolutie van antibioticaresistentie

Fenotypische veranderingen spelen een centrale rol spelen in het aanpassingsvermogen van bacteriële populaties en daarom de natuurlijke selectie aansturen. In deze context gaan populaties die zich aan omgevingsverstoringen aanpassen met een voortplantingsvoordeel gaan domineren. Tijdens de druk van antibiotica kunnen erfelijke mutaties die de gevoeligheid sterk verminderen zich snel verspreiden. Echter gaat dit gepaard met ingrijpende veranderingen in essentiële cellulaire processen die de fitheid van de cellen verlagen. Daarom gaan de resistentie-mutaties vaak samen met ondersteunende aanpassingen om dit voorplantingsnadeel verminderen. Metabole aanpassingen zijn hier onderdeel van, als de voedingsstoffen in de

omgeving dit toelaten. Daarnaast wordt het steeds duidelijker dat metabolisme-gerichte mutaties ook een klasse van resistentiemechanismen vormen. Hierdoor kunnen de nutriënt- en stofwisselingscontext de evolutie van resistentie actief mede sturen.

Hoofdstuk 5 beschrijft de metabole aanpassingen tijdens resistentie-evolutie en hoe metabolomics kan helpen die te beter te begrijpen. Omdat één methode niet alle kleine moleculen kan meten, vraagt metabolomics om gerichte keuze van analysetechnieken en monstervoorbereiding passend bij de onderzoeksvraag. Omdat het metabolisme sterk kan verschillen tussen bacteriestammen en aanpassingen per antibioticaklasse anders zijn, maakt dit de keuze wel ingewikkeld. Daarnaast is de keuze nog beperkt, wat wordt weergegeven door de 23 geïncludeerde studies in de review uiteenlopende bacteriën en antibioticaklassen bestrijken. Dit benadrukt dat er systematischer metabolomics-onderzoek nodig is die vooral focussen op een juiste combinatie van biologisch vraagstuk en analytische methodologie. Uiteindelijk kan de rol van metabolisme in antimicrobiële resistentie beter te verklaard worden met deze analytische methodes.

In **Hoofdstuk 6** is getest hoe de resistentie-evolutie van *P. aeruginosa* tijdens adaptieve laboratoriumevolutie plaatsvindt in kweekmedium met een enkele nutriënt. Na deze evolutieperiode van 10 dagen zijn de fenotypische en genotypische veranderingen onderzocht. De geëvolueerde lijnen vertoonden significante verschillen in MIC tussen de verschillende enkelvoudige-nutriëntcondities. In het experiment werden ook mutaties gedetecteerd die uniek waren voor specifieke evolutiecondities, maar slechts zes mutaties in totaal over de 15 onderscheiden condities waarvan het DNA was gesequencet. Ook is er maar een kleine set van mutatiepatronen gevonden die de evolutiecondities betrouwbaar van elkaar onderscheidde. De smalle bandbreedte van deze mutatieverandering loopt parallel aan de vrijwel identieke groeisnelheidsverschuivingen na evolutie binnen elke antibioticaklasse. Deze beperkte variatie in fitness stuurt populaties richting een beperkte set genotypen.

De diversiteit manifesteerde zich dus vooral als MIC-verschuivingen, niet als uitgesproken mutatieprofielen. Omdat *P. aeruginosa* langdurig in CF-longen kan persisteren, kunnen zulke fenotypische verschuivingen die al na 10 dagen

optreden op termijn ook vestigen in stabiele genotypische verschillen. Maar met deze kennis dat enkele nutriënten de resistentieontwikkeling veranderen kan een volgende stap om te onderzoeken hoe verschillende infectierelevante nutriëntcondities de ontwikkeling van resistentie sturen genomen worden.

Samenvattend liet Sectie III zien dat metabole processen een cruciale rol spelen in de ontwikkeling van antibioticaresistentie en dat nutriënten een regulerende invloed uitoefenen ondanks de metabole veelzijdigheid van *P. aeruginosa*. Echter is de invloed van de samenstelling van het kweekmedium slechts een klein gedeelte van de complexe evolutionaire trajecten die in de CF-long worden waargenomen. Niettemin wijzen de resultaten erop dat de invloed van de nutriëntomgeving antibioticumafhankelijk is. Dit benadrukt diepgaander onderzoek naar de onderliggende aanpassingen die tijdens antibioticablootstelling optreden.

8.6. Vervolgstappen

Antibiotica zijn onmisbaar in de dagelijkse gezondheidszorg om infecties te bestrijden, toch zijn er groepen patiënten waarbij infectieziekten nog een groot probleem veroorzaken. Daarnaast is fungeert de lage kans van slagen van de antibioticabehandeling in de patiëntengroep als een reservoir voor antibioticaresistente stammen. Het bestuderen van de bacteriële aanpassingen in deze patiëntengroep geeft cruciale informatie over de uiteenlopende manieren waarop antibiotica therapieën kunnen falen. De luchtwegen van CF-patiënten vormen zo'n unieke omgeving. In deze omgeving groeit de doorgaans onschuldige bacterie *P. aeruginosa* uit tot een van de meest uitdagende infecties om te behandelen.

Een sleutel factor van *P. aeruginosa*-infecties in de CF-long is de specifieke nutriëntomgeving. Dit is een aspect dat vaak over het hoofd wordt gezien. De nutriëntomgeving kan het bacteriële gedrag diepgaand beïnvloeden en leiden tot misinterpretatie van gevoeligheidstesten wanneer deze met standaard kweekmedia worden uitgevoerd. Daarom is het essentieel om te focussen op: (I) het in kaart brengen van fysiologisch relevante infectielocaties, (II) het onderzoeken van de invloed van nutriënten op antibioticagevoeligheidstesten, (III) het vastleggen van door nutriënten geïnduceerde veranderingen in

antibioticagevoeligheid, en (IV) het ontrafelen van de onderliggende biologische mechanismen die verantwoordelijk zijn voor veranderingen in het antibiotisch effect.

Het beter in kaart brengen van welke voedingsstoffen in welke delen van de CF-long aanwezig zijn is belangrijk omdat dit proefschrift laat zien dat voedingstoffen een grote rol spelen de PD van antibiotica. Het is daarbij goed om te beseffen dat niet alle monsters die uit de CF-long worden genomen dezelfde informatie geven. Sputum is het makkelijkst te verzamelen, maar bestaat uit materiaal uit verschillende delen van de long. Hierdoor geeft het alleen een gemiddeld beeld van de chemische samenstelling. Andere methoden die vocht verzamelen van het longoppervlak met speciale instrumenten zijn specifieker, maar ook lastiger uit te voeren met een minder nauwkeurige bepaling van de voedingsstoffen. Daarnaast zijn er ook biofilms, die binnen de biofilm-structuur verschillende micro-omgevingen hebben.

In de oncologie is veel vooruitgang geboekt in het begrijpen van zulke complexe structuren door het gebruik van 'tumoratlassen'. In de tumoratlassen wordt informatie gecombineerd over celtypes, genetische veranderingen en de lokale omgeving. Deze kennis wordt inmiddels ook gebruikt om patiënten gerichter te behandelen. Voor infecties in de CF-long zouden we op eenzelfde manier ook met geïntegreerde kaarten van de infectieomgeving gaan werken. Hierbij kunnen moleculaire, microbiële en klinische gegevens worden gecombineerd voor het onderscheiden van verschillende typen longomgevingen. Deze onderscheiding tussen patiënten is de basis om het effect van voedingstoffen op de werking van antibiotica te begrijpen.

Om beter te voorspellen hoe antibiotica werken in de CF-long, moeten de in vitro testen aangepast worden om de infectieomstandigheden beter te imiteren. Dit proefschrift laat zien dat zelfs kleine veranderingen in de voedingsstoffen de effectiviteit van antibiotica beïnvloeden. Een volledige hervorming van klinische testmethodes is lastig, maar stappen vooruit zijn mogelijk. Hierbij is het in eerste instantie noodzakelijk om de bovengenoemde omgevingen goed in kaart te brengen. Maar er kunnen al veranderingen gemaakt worden zoals het testen van bacteriën uit verschillende delen van de long, of door ook anaerobe condities te gebruiken voor een van origine aerobe bacterie als *P. aeruginosa*.

Bacteriestammen passen zich door langdurige blootstelling aan condities aan, waardoor de stammen moeilijk te kweken zijn in het laboratorium. Toch kunnen deze gespecialiseerde stammen een factor spelen in therapiefalen wanneer ze in de niche omgeving van de patiënt zitten.

Om beter te begrijpen hoe voedingsstoffen de werking van antibiotica beïnvloeden, is in dit proefschrift gekozen voor time-kill experimenten en farmacodynamische modellen. De resultaten uit dit proefschrift laten zien dat een standaard MIC-test grote verschillen in groeisnelheid en afsterven van bacteriën kan verbergen. Daarom is het belangrijk om vervolgonderzoek naar de invloed van voedingsomstandigheden kritisch te kijken naar uiteindelijke read-out van de experimenten. Zo heeft ook de sigmoïde concentratie-effectcurve die in dit proefschrift gebruikt is om combinaties van antibiotica en voedingsstoffen in kaart te brengen ook beperkingen. De effectdata die zijn gebruikt voor het ontwerp van het farmacodynamisch model zijn verkregen uit een statische time-kill experimenten. Deze methodologische aanpak kan bacteriële aanpassingen tijdens de blootstelling aan antibiotica slecht nauwkeurig vastleggen. Deze beperkingen benadrukken de noodzaak van dynamische *in vitro* kweeksystemen, die beter inzicht kunnen geven in tijdsafhankelijke farmacodynamische kenmerken zoals veranderende groeisnelheden, dodingskinetiek en resistentieontwikkeling.

Een belangrijk inzicht uit dit proefschrift is dat het effect van antibiotica sterk afhangt van de combinatie met specifieke voedingsstoffen. Zulke voedingsstof-antibioticumcombinaties kunnen unieke aanpassingen in bacteriën veroorzaken, die niet altijd verklaard worden door bekende resistentie-mutaties in genetische databanken. Om deze mechanismen beter te begrijpen zijn technieken zoals transcriptomics, proteomics en metabolomics nodig. Deze brengen veranderingen in genexpressie, eiwitten en metabolieten in kaart en geven zo beter inzicht in hoe bacteriën reageren op antibiotica. In Hoofdstuk 5 is bijvoorbeeld beschreven hoe metabolomics kan worden ingezet om antibiotica-gevoeligheid te verbeteren door gerichte nutriënten toe te voegen tijdens de behandeling.

Een andere veelbelovende techniek is fluxomics, die laat zien hoe metabolieten en energie door cellen stromen. Hoewel dit waardevolle inzichten

oplevert tijdens stress of antibioticadruk, is de brede toepassing op klinische stammen beperkt omdat de modellen te complex en specifiek zijn. Om de extrapolatie tussen de verschillende stammen te verbeteren kunnen geavanceerde computermodellen gebruikt worden die meerdere -omics datasets combineren. Deze modellen geven een breder beeld van de veranderingen over tijd zoals nutriëntenschommelingen en resistentieontwikkeling. Ook zijn er single-cell technieken in opkomst om verschillen binnen bacteriepopulaties te begrijpen. Deze technieken zijn waardevol, maar moeten ingebed zijn in bredere analyses om goed te interpreteren hoe bacteriële heterogeniteit bijdraagt aan behandelresistentie.

7.3. Conclusies

Concluderend laat dit proefschrift zien dat de beschikbaarheid van voedingsstoffen en zuurstof een grote invloed heeft op hoe *P. aeruginosa* zich gedraagt en de antibiotica farmacodynamiek. Een antibioticabehandeling is dus niet alleen afhankelijk van het medicijn en de bacterie, maar ook van de omgeving waarin de bacterie zich bevindt. Dit geldt vooral voor complexe infecties zoals in de luchtwegen van CF-patiënten.

De bevindingen in dit onderzoek vormen een belangrijke stap naar het verbeteren van antibioticatesten in het laboratorium, door daarbij beter rekening te houden met realistische omstandigheden zoals zuurstofgebrek of specifieke nutriënten. De volgende uitdaging is om deze kennis te koppelen aan de situatie in het lichaam van de patiënt, zodat behandelingen beter afgestemd kunnen worden op de plek van de infectie. Zo komen we dichter bij antibiotica behandelingen op maat.

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Appendices

Curriculum vitae

Maik Kok was born on 18 November 1994 in Rotterdam, the Netherlands. He completed secondary school at Penta Scala Molenwatering in 2013 and went on to study Bio-Pharmaceutical Sciences at Leiden University, earning his bachelor's degree in 2016. He then pursued the master's in Bio-Pharmaceutical Sciences at Leiden University.

His master's program began with a nine-month internship in the Division of Analytical Biosciences, now the Metabolomics and Analytics Center (MAC), at the Leiden Academic Centre for Drug Research (LACDR), supervised by Dr. Amy Harms and Dr. Cornelius Willacey. During this project, he developed a sensitive analytical method to quantify dopamine and related neurotransmitters in in vitro neuronal cells. He next completed a six-month internship at the Algemeen Klinisch Laboratorium of the Netherlands Cancer Institute in Amsterdam, supervised by Dr. Huub van Rossum and Dr. Lennart van Winden. There, he established a robust analytical method for estrogens that has since been implemented in clinical practice for the diagnosis and prognosis of HR-positive breast cancer. Maik graduated cum laude with his master's degree at the end of 2018.

In 2019, he started his PhD as part of a multidisciplinary project between the Division of Systems Pharmacology and Pharmacy and the Metabolomics and Analytics Center at LACDR, supervised by Prof. Dr. Coen van Hasselt and Prof. Dr. Thomas Hankemeier. His research focused on how nutrient environments shape the antibiotic pharmacodynamics of *Pseudomonas aeruginosa*. Alongside his research, his contributions to teaching and mentoring led to the award of the University Teaching Qualification during his PhD.

Maik currently works at ZonMw in The Hague as a program secretary, facilitating funding for research and innovation in health, healthcare, and well-being.

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Leiden, The Netherlands

LACDR spring symposium 2021 – poster presentation

Online

Metabolomics 2019 – poster presentation

The Hague, The Netherlands

LACDR spring symposium 2019 – poster presentation

Leiden, The Netherlands

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Jullie maken het verschil!

“komt goed” – Maik Kok