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

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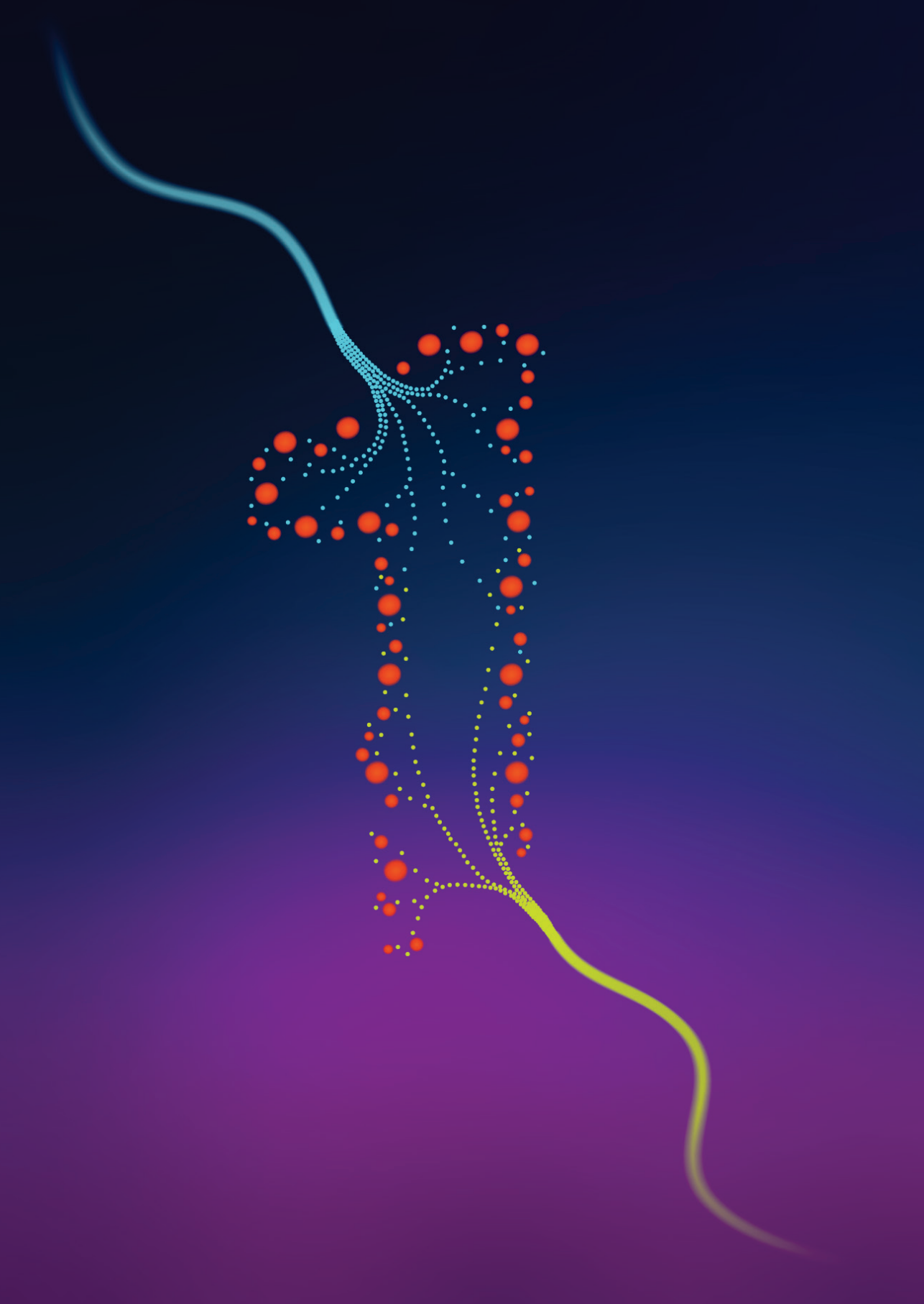

# Section I

## 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

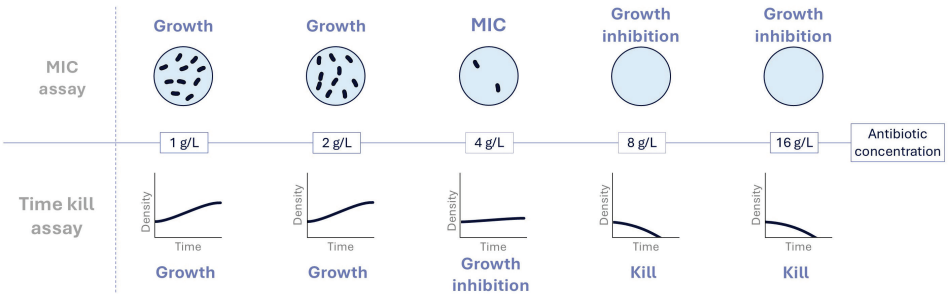


**Figure 1.** Difference between read-outs to evaluate the effect of antibiotics on bacterial growth and/or kill using minimal inhibitory concentration (MIC) and time-kill assays. MIC assays evaluate antibiotic drug effects at a fixed time point and can only assess growth inhibition. Time kill assays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

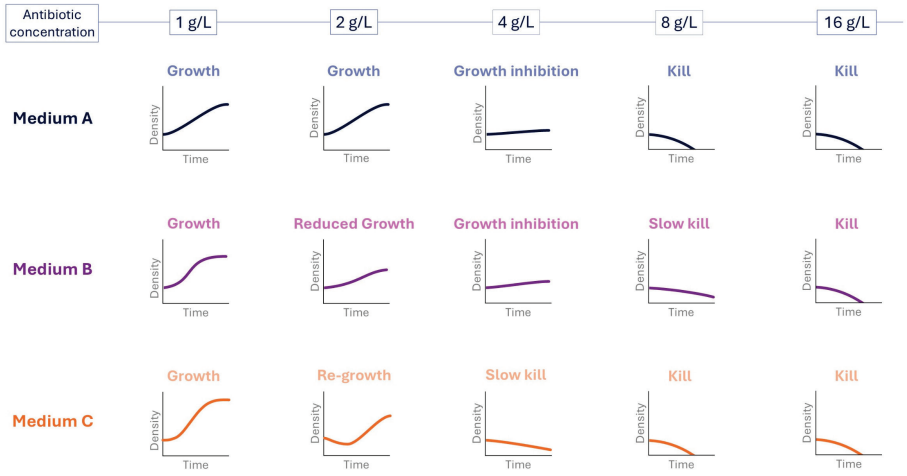


**Figure 2.** Examples illustrating the effect of media/nutrient conditions on phenotypic responses to antibiotic exposure. Although the dynamic responses differ, these conditions would yield similar outcomes in minimal inhibitory concentration (MIC) assays.

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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