MASTER'S THESIS

IMPACT OF THE TYPE VI SECRETION SYSTEM AND AMPDH3 AMIDASE ON *PSEUDOMONAS AERUGINOSA*'S CAPACITY TO COMPETE WITH CYSTIC FIBROSIS-RELATED NON-FERMENTING GRAM-NEGATIVE BACILLI

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Master's Degree in Advanced Microbiology

(Specialisation/Pathway *Research in Sanitary Microbiology*)

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Academic Year 2021-22

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Key words:

Pseudomonas aeruginosa, AmpDh3, Type VI secretion system, *Burkholderia multivorans, Stenotrophomonas maltophilia, Achromobacter xylosoxidans*, cystic fibrosis, competition.

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ABSTRACT

The need for novel antipseudomonal therapies is driven by the severe clinical-epidemiological threat posed by *Pseudomonas aeruginosa*'s rising levels of acquired antibiotic resistance, in the field of nosocomial opportunistic infections and in the unique context of Cystic Fibrosis (CF)-associated chronic infections. Identifying therapeutic targets is the first essential step in the process of drugs development, and in this regard, to deeply understand how different species can coexist and compete to occupy the CF niche, where *P. aeruginosa* is an extraordinarily successful species, could be an interesting source of therapeutic targets. For this reason, this study sought to better understand the importance of an enzyme, the peptidoglycan amidase AmpDh3, recently described to be essential for *P. aeruginosa* competition capacity against certain species not related to chronic niches. Thus, the role of AmpDh3 toxin but also of the type VI secretion system *per se* (T6SS, the mechanism through which this toxin is injected in competitor cells to degrade their peptidoglycans) were analyzed in terms of their importance for the competition capacity of *P. aeruginosa* versus typical rival species in the CF niche. To do so, in this work simple mutants deficient in *ampDh3* and in *retS* (repressor of T6SS), and a double KO strain were used, in order to analyze the resulting *P. aeruginosa* phenotypes in the competition carried out against *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia multivorans* (gram-negative species described to be a notable cause of chronic infection in CF patients) in solid medium.

Our results indicate a variable importance of T6SS *per se* in the competition capacity of *P. aeruginosa* against the mentioned species: greater against *S. maltophilia*, much milder against *A. xylosoxidans* and despicable against *B. multivorans*. Meanwhile, although our results suggest that AmpDh3 slightly contributes to *P. aeruginosa* competitiveness against these species when the T6SS is de-repressed, its importance seems much minor compared to previous works in which it was shown to be essential to challenge species not related with CF. Future work will be needed to fully understand these facts and their potential therapeutic implications.

INTRODUCTION

1. General characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (PA) belongs to the *Pseudomonas* genus, located within the *Pseudomonadacaeae* family, *Gammaproteobacteria* class and *Proteobacteria* phylum, and is the main pathogenic species of this genus, displaying an extraordinary clinical relevance, topic approached below (García-Valdés & Lalucat, 2016). PA is a strictly aerobic, metabolically versatile, oxidase and catalase-positive Gram-negative spores non-producing bacillus. Its cells are usually straight, 0.5 to 1 μm in diameter, 1.5 to 5 μm long, and generally mobile thanks to the presence of a polar flagellum, although it is not exceptional to find isolates bestowed with two or three flagella, as well as strains that lack this element due to mutations. Furthermore, the colonies of PA are usually extended, sparkly and smell like grapes or tortilla chips (Wu et al., 2015).

PA has the ability to grow between 4ºC and 42°C, which differentiates it from many other *Pseudomonas* species; however, its optimal growth temperature is 37°C and in the laboratory it can flourish in both enriched and non-enriched culture media (Wu et al., 2015). PA and the rest of the members of the genus' fluorescent group (*P. fluorescens*, *P. putida*, *P. veronii* and *P. monteilii*) produce pigments that are fluorescent under UV light, including pyoverdine, the main water-soluble siderophore of the *Pseudomonas* genus that appears yellow-green in colour (Meyer, 2000). Other pigments that can be produced by PA depending on the strain, include pyocyanin, a non-fluorescent blue-green pigment, pyorubin (reddish colour), and pyomelanin, a brown pigment that usually appears in strains isolated from chronic lung infections (Rodríguez-Rojas et al., 2009). PA can be found in a wide range of environmental habitats, but due to its propensity for moist environments, it is also usual its isolation from human body parts such as the nasal mucosa and pharynx without causing infection; in other words, acting just as a colonizer. Thus, this species can colonize and flourish in a variety of inert surfaces, settings and living beings (plants, animals, and, as already noted, humans) thanks to its extraordinary metabolic versatility and adaptation capacity (Crone et al., 2020). Additionally, it is possible to isolate PA from aqueous solutions used in medical facilities, including soaps, antiseptics, eye drops, ventilation systems, dialysis fluids, mechanical breathing devices, swimming pools, cosmetics, fruits, and vegetables, among others (Tripathy et al., 2007). These features and its great success for dissemination within the human environment makes very difficult the eradication of *P. aeruginosa* from hospital settings and other sanitary institutions, which supports its role as one of the most common causes of

nosocomial infection. The PA capacity for antibiotic resistance development is also a hallmark of this species, as will be shown below. Just to provide an idea, its prevalence of resistance often displays extraordinary levels, such as resistance to imipenem reaching up to 42%, meropenem at 46%, or tobramycin at more than 50% of isolates, in some geographical areas such as certain Mediterranean countries or southeast Asian ones (El Zowalaty et al., 2015). This feature has not only obvious clinical consequences, but also dramatic economic impacts, with huge associated healthcare costs: it has been estimated that if no new effective antibiotics are introduced, by 2050 the worldwide increases in healthcare expenditures related to antibiotic resistance may reach between US$300 billion and US$1 trillion annually. Moreover, it has been also estimated that by 2050, if the current trend of growing resistance is not disrupted, the deaths associated to antibiotic-resistant infections (PA is the species with greatest impact in this sense) will reach 10 million/year, clearly evidencing the size of the problem (Ahmad & Khan, 2019).

2. Clinical significance of *Pseudomonas aeruginosa*

PA is one of the leading causes of opportunistic infections in humans and is regarded as the most significant human pathogen within its genus, both for the number and variety of diseases that it causes as well as their morbidity and mortality (Aloush et al., 2006). As usually occurs with the bacterial opportunistic pathogens, PA has its greater impact as cause of healthcare-associated infections, being therefore considered one of the top nosocomial pathogens. The origin of the opportunistic PA infections can be the own colonizer strains of the patient and/or their dissemination from other individuals or environmental sources, that finally develop the infection under certain conditions.

This pathogen can cause a great variety of infections, from superficial and mild ones to bacteremia and other severe ones that can endanger the life of patients. The eye, skin (in burns), respiratory tract, and urinary tract infections are the most common, but PA can attack almost all anatomical regions and in certain situations, disseminate to cause bacteremia, sepsis, and even septic shock. Patients with immunodeficiencies and/or those subjected to invasive procedures (mechanical ventilation, catheters, surgery), or showing severe burns or ulcers are typically the most vulnerable, because of this disruption of natural anti-infective barriers that they suffer (Richards et al., 1999). These infections, among which the ventilator-associated pneumonia stands out because of its great incidence, are categorized as acute, invasive, cytotoxic, and sometimes resulting in systemic spread, having the potential to cause septic shock and high mortality.

In accordance with all these facts, it is not a surprise that PA is one of the top pathogens causing infections in critical patients, i.e., those admitted to Intensive Care Units (ICUs), that obviously often display several of the mentioned predisposing factors. PA is also

capable of causing some community-acquired infections with generally low prevalence/importance, but in this context the pathogen has a very important role as the first cause of chronic infection of patients with underlying chronic respiratory diseases such as cystic fibrosis (CF), or to a lesser extent, chronic obstructive pulmonary disease (COPD). These chronic infections are non-cytotoxic, not particularly invasive, and do not evolve to systemic spread, but they can cause a progressive and finally fatal damage to the lung's epithelium. The chronicity of these infections is intimately associated to PA's capacity to form biofilms and to show decreased expression of genes linked to virulence, among many other adaptive features that acquires along time during the chronic process (see below) (Brewer et al., 1996; Rajan & Saiman, 2002; Cantón et al., 2015).

Since PA's virulence cannot be traced to a single factor, its pathophysiology is frequently characterized as multifactorial. As a result, most strains produce surface and soluble virulence factors that allow adhesion, colonization, and invasion, which cause tissue injury or trigger the production of inflammatory cytokines, among other effects. Obviously, these factors pose one of the bases for the clinical importance of this species, and in the next paragraph a brief overview of the most important representatives is shown.

2.1 Virulence factors

PA displays a wide variety of virulence factors that enable the abovementioned variety of infections. Chronologically speaking, those acquiring more importance in the initial stages of infection for obvious reasons are the adhesins (pili and lipopolysaccharide, LPS), which help to colonize the epitheliums through specific receptors. The LPS has other functions besides adhesion, as happens in other Gram-negative species, such as the defense against humoral immune components (complement, antimicrobial peptides, etc.). Then comes the flagellum, which allows PA to move by swimming and swarming, as well as the adhesion to tissues (Feldman et al., 1998). Toxins are another very relevant virulence factor, since they cause host's cells death facilitating the penetration of PA into the tissues, but also because the targeted cells can be even the host's phagocytes, with obvious advantages for the bacterium. ExoS, ExoT or ExoU, are examples of exotoxins found in PA that are transferred directly from bacterial cytoplasm to that of the host cell (thus avoiding antibody action and degradation) by means of the type III secretion system (T3SS), also known as the injectisome and typically conserved in certain bacterial pathogens (Galán & Collmer, 1999). These exotoxins cause intracellular disorders by altering the actin cytoskeleton and specific signaling pathways, which end up facilitating cell death (El-Solh et al., 2012). Moreover, the type VI secretion

system (T6SS), which has a structure and mechanism somehow like that of T3SS, helps PA to survive by transferring other protein effectors into the cells of the host, but perhaps more importantly, also of other bacterial competitors, causing their death. Additionally, T6SS also takes part in the PA biofilm development capacity (Chen et al., 2015). Besides, PA produces hemolysins, phospholipase C (Plc), and rhamnolipid (Rhl), which mostly play a role in the lungs of CF patients by acting at the alveoli level causing their collapse, accounting for the progressive loss of respiratory function (Soberón-Chávez et al., 2005). In PA, siderophores such as pyoverdine and pyochelin enable the obtainment of iron within the host (a clear limiting factor for infection), whereas the production of the exopolysaccharide alginate (and to a lesser extent, of other polymers such as Pel and Psl), is an essential element for biofilm formation, acting as a physical barrier that prevents the arrival of antibiotics and immunity elements (Boyd & Chakrabarty, 1995; Meyer, 2000). The biofilm mode of life acquires special relevance in the chronic respiratory infections such as those caused by PA in CF patients, as will be shown below.

2.2 Antibiotic resistance

The most striking feature of PA, that decisively contributes to its clinical relevance, is its great capacity for developing antibiotic resistance, whose mechanistic basis is outlined next. In general, the resistance of PA (just like any other bacteria) is due to the combination of two factors, namely the intrinsic and the acquired resistance. The former is naturally encoded in its genome and is primarily dependent on the great impermeability of its outer membrane (to which a wide variety of basally produced efflux pumps contribute) and the expression of the inducible chromosomal β-lactamase AmpC, which confers resistance to a notable number of β-lactam antibiotics (e.g., amoxicillin, ampicillin, cefoxitin, etc.). Meanwhile, the acquired resistance may appear due to two causes: 1) selection of chromosomal mutations that can affect a variety of elements; for instance, the antibiotic's target (if the target changes, the antibiotic cannot recognize it, as happens with fluoroquinolones, whose target are the DNA gyrases/topoisomerases); mutations that cause the loss of membrane porins (such as OprD) through which the antibiotics such as carbapenems should naturally enter the cell; mutations that cause the constitutive hyperexpression of the abovementioned efflux pumps (mainly MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) or the β-lactamase AmpC, which boost the resistance capacity provided by their basal expression. 2) By horizontally-acquired resistance determinants, mostly through plasmids (conjugation), but also by transduction and/or transformation processes. Among these determinants, the most relevant are the β-lactamases, which may come in a variety of forms, but those known

as "extended spectrum" or the carbapenemases, clearly stand out because they confer resistance to the most recent antipseudomonal β-lactams (extended spectrum cephalosporins and carbapenems). Besides, different aminoglycoside-modifying enzymes are also often horizontally-acquired and are typically encoded together with β-lactamases in genetic elements known as integrons (El Zowalaty et al., 2015).

Thanks to the mentioned mechanisms, PA is one of the species with higher capacity for resistance development if not the top one, and in fact, clinical strains showing resistance to all the families of antibiotics (known as pan-drug resistant) have been reported. These facts are making the current antipseudomonal arsenal progressively less effective, and thus, new therapeutic solutions are needed. In this sense, although new drug combinations such as ceftolozane/tazobactam, imipenem/relebactam, ceftazidime/ avibactam, etc., are progressively being introduced into clinical practice, PA has already shown its capacity to acquire resistance against them. Thus, it is necessary to approach new strategies, and among them, those aimed at interfering with known resistance mechanisms (to rehabilitate classic antibiotics) and/or at attenuating the virulence of the pathogen are gaining interest in the last years to face a great adversary as is PA (Mancuso et al., 2021; Yahav et al., 2021).
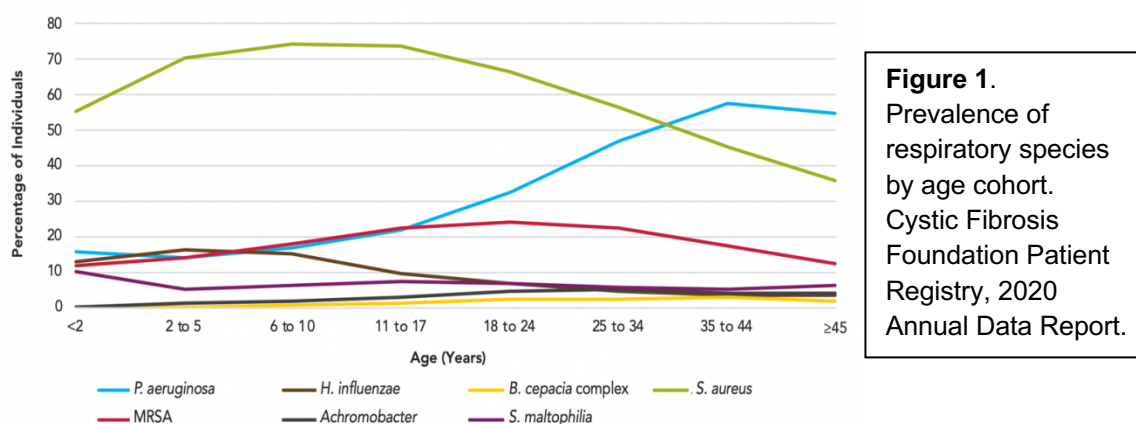
3. *Pseudomonas aeruginosa* and its role in CF-associated chronic pulmonary infection

Severe respiratory infections are uncommon in healthy people because of sophisticated immune defense systems at the lung mucosa. The first line of defense against bacteria is made up of bronchial and alveolar epithelial cells in the airways. These cells not only act as a physical barrier and display local antimicrobial activity (mediated by secreted humoral compounds such as antimicrobial peptides), but also act as sentinels, encouraging the recruitment and activation of immune cells (mainly neutrophils) that destroy bacterial invaders after a controlled inflammatory reaction, elicited by the cytokines released by them. As important mediators of innate and adaptive immunity, resident macrophages and often dendritic cells are also present in the alveolar epithelium. Contrarily, patients with chronic respiratory underlying diseases like CF, bronchiectasis or COPD have impaired immune responses within the respiratory airways; as a result, and in combination with other underlying mechanisms explained below, these disorders are marked by recurrent cycles of inflammation, tissue damage, and bacterial growth that eventually result in the establishment of chronic and irreversible respiratory infections and a rapid decline in pulmonary function (Döring et al., 2011; Eisele & Anderson, 2011).

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Multiple mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, a cAMP-regulated chloride channel in epithelial cells, can be the cause of CF. Chloride transport at the apical surface of epithelial cells is compromised when CFTR is dysfunctional because of a lack of synthesis, a failure of the protein to reach the site of action on the apical membrane, or because of other functional flaws. Even though the CFTR protein is expressed in numerous organs, malfunction in the respiratory, gastrointestinal and reproductive systems predominates in the clinical picture of CF patients. Over 90% of these patients die from pulmonary illness, which also accounts for most CF-related morbidities (Davies, 2002). The usual PA establishment and infection in CF patients is a complex phenomenon governed by several circumstances, being among them the mutant CFTR's failure to efficiently release chloride from respiratory epithelial cells into the liquid at the surface of the airway, which leads to an increased water absorption driving to an increased viscosity of the mucus (thus acting as a bacterial trap) that also shows alterations in the concentration of salts. On the other hand, this viscosity impairs ciliary clearance and may hinder the uptake of antimicrobial peptides onto the epithelium, entailing a decrease on its antimicrobial properties. The mentioned viscosity could also have an adverse effect on neutrophils' and other immune cells' movement towards the pathogen, as well as making more difficult the diffusion of humoral immune elements. Furthermore, besides a poor diffusion of gases in these conditions, bacterial pathogens colonizing the lung consume oxygen within the highly viscous mucus, creating an anaerobic or microaerobic environment that prevents neutrophils and other immune cells from acting efficiently to eliminate bacteria. Last but not least, CFTR deficiency may also contribute to PA's chronic infection, since in a functional condition, it mediates, after interaction with LPS, the internalization into host cells. However, when CFTR is altered (as happens in CF), the bacterial LPS does not efficiently bind to it, favoring the bacterial permanence on the surface of the epithelium (thus contributing to biofilm formation) instead of invading it (which would be more typical of an acute process) (Döring et al., 2011).

All the aforementioned features of the CF airway create the perfect conditions for colonization and infection due to PA, but also other species. In this sense, although *Staphylococcus aureus* and to a lesser extent, *Haemophilus influenzae*, are typically isolated in young children with CF, patients actually experience different predominance of bacterial species throughout their lifetime. For instance, other opportunistic multidrug resistant pathogens like *Achromobacter* spp., *Burkholderia cepacia* complex (BCC) species or *Stenotrophomonas maltophilia*, are significantly isolated during adolescence

and, especially PA is very often found during adolescence and early adulthood onwards, whereas the prevalence of *S. aureus* tends to decline (Figure 1).

Figure 1. Prevalence of respiratory species by age cohort. Cystic Fibrosis Foundation Patient Registry, 2020 Annual Data Report.

Thus, most adult CF patients (about 60%) are chronically infected with PA, being the infection very difficult to eradicate due to the progressive antibiotic resistance acquired by the bacterium. Interestingly, it is usual in CF that the chronic colonization is caused by the same PA strain during long periods (even years) in a single patient, which is clearly associated with its adaptation to the CF niche. During this chronic process, PA can show long periods in which it seems dormant, which are interspersed with punctual episodes of acute overgrowth associated with increased inflammation and decreased pulmonary function (known as exacerbations), threatening the patient's life. PA expresses a variety of virulence factors that allow it to evade the host's defenses and apparently are more important in the CF than in the acute infection context, including certain lipases, proteases, rhamnolipid, pyocyanin, quorum sensing (QS) molecules, catalases, and exopolysaccharides (such as the biofilm-forming alginate). Some of these elements are directly harmful for the lung tissue, although they also act by impairing immune cell activity. Among these factors, and mostly during the initial stages of chronic establishment, those affecting neutrophils' activity (e.g., pyocyanin, alkaline protease, exotoxins) are crucial because an effective PA clearance depends on neutrophil recruitment, phagocytosis, and the generation of neutrophil-derived anti-microbials (i.e., granular contents such as lysozyme and antimicrobial peptides). Finally, and in addition, even though there is a large influx of neutrophils into the infected CF lung, the CFTR-dependent neutrophil activity impairment (such as decreased phagosome chlorination) makes this recruitment highly ineffective, increasing CF patients' susceptibility to PA (Goncalves-de-Albuquerque et al., 2016). These facts trigger a continuous excessive but ineffective recruitment of neutrophils, that end up releasing certain intragranular

compounds (such as proteases and lipases) resulting in harmful effects for the host tissues, which contributes to the pathogenesis of CF.

In the CF context PA faces numerous environmental stresses during chronic infection of the airway, including interspecies competition for nutrients and space, anaerobic/ microaerobic environment brought on by mucus plugging, high concentrations of salts and antibiotics used to treat the infection, and a surplus of antibodies, neutrophils and neutrophil-derived antimicrobials (such as antimicrobial peptides and reactive oxygen species, ROS). PA undergoes microevolution along the chronic process, which is characterized by the acquisition of spontaneous mutations and the selection of phenotypically different variations that are better adapted to such particular niche. Thus, PA changes from an "early/acute" to a "late/chronic" behavior in CF thanks to this evolutionary process. Some phenotypic traits connected to this transition are the appearance of hypermutator strains and the upregulation of exopolysaccharide expression (Psl/Pel and alginate) which promotes the formation of more robust biofilms. Biofilms are sessile bacterial communities surrounded by thick layers of alginate and mucus, in which usually appear bacterial microcolonies or clumps that are closely surrounded by neutrophils and the airway epithelium. As previously stated, biofilm mode of life poses a physical barrier, and consequently, PA growing in biofilms promotes less complement system activation and additionally, when neutrophils reach biofilms, the immune cells become stuck at the top levels of the microcolonies and are unable to effectively clean the bacterial communities (Goncalves-de-Albuquerque et al., 2016; Malhotra et al., 2019; Welp & Bomberger, 2020). Moreover, the biofilm matrix also possess a handicap for the correct diffusion of antimicrobial treatments administered to the patients, that besides the progressive selection of mutations driving to antibiotic resistance acquired by the chronic strains, make the therapeutic weapons highly unsuccessful to treat the chronic infection caused by PA in CF patients (Rossi et al., 2021).

Other features that are seemingly selected during PA adaptation to CF are the downregulation of some virulence factors, that seem expendable for the chronic persistence. For instance, it is usual the loss of activity of the motility appendages pili and flagellum, as well as certain modifications of LPS. Even though PA flagellar motility is required for initial colonization of the host, the flagellum is a PAMP (Pathogen Associated Molecular Pattern) recognized by the epithelium and phagocytes; as a result, downregulating the expression of flagella during chronic infection apparently enables

evasion of immune cell recognition and phagocytosis while also decreasing the production of pro-inflammatory cytokines/chemokines. Similar trends have been attributed to the LPS (that accumulates changes driving to a less immunogenic capacity), but also to T3SS, also tending to be selected for downregulation. This circumstance is favored by the fact that, once protected by the biofilm, PA cells can dispense with this system for toxin secretion since they have less need to invade epithelia and/or kill phagocytic cells (Rossi et al., 2021). Altogether these features and adaptations make of PA one of the most successful pathogens causing infection in CF patients, although other species can also compete/co-exist to occupy the niche.

4. Other non-fermenting gram-negatives causing chronic infection in CF

Other less frequent non-lactose fermenting Gram-negative species have become significant pathogens in a notable proportion of CF patients, including, among others, *Stenotrophomonas maltophilia*, BCC species (e.g., *Burkholderia multivorans*) and *Achromobacter xylosoxidans*. These organisms are found in abundance throughout nature, especially in soil and water sources, but similarly to *P. aeruginosa*, they have successfully adapted to human environments, displaying an important capacity to act as opportunistic pathogens. They are also typically challenging to eliminate from healthcare facilities because they have evolved to live in a variety of conditions. And also, similar to PA, they frequently exhibit broad antibiotic resistance due to several intrinsic/acquired resistance mechanisms, including drug efflux pumps and intrinsic and/or horizontally-transmitted β-lactamases (Spencer et al., 2020). The main characteristics of each of these species, that in any case are much less prevalent in CF than PA, are summarized in the next paragraphs.

S. maltophilia (SM) is an aerobic, oxidase-negative, catalase-positive, opportunistic Gram-negative pathogen, able to cause a wide variety of acute infections, such as keratitis, endocarditis, meningitis, acute respiratory tract infection, bacteremia (with/without hematological malignancies), tropical pyomyositis, or septic arthritis. However, it also acquires a notable relevance as cause of chronic infection in CF patients. In these individuals, a higher risk of pulmonary exacerbations and hospitalizations has been shown, but different studies and investigations have found varying degrees of lung function decrease associated to different strains of SM (Menetrey et al., 2021). The prevalence of SM infections in CF patients has remained consistent at around 12 percent, according to the U.S. Cystic Fibrosis Foundation Annual Data Reports (Adegoke et al., 2017).

The species belonging to the BCC, e.g., *B. multivorans,* (BM), *B. cenocepacia, B. cepacia, B. pyrrocinia, B. vietnamiensis,* etc., are Gram-negative, aerobic, catalase and oxidase-positive bacteria. One of the most common BCC species is BM, responsible for between 85% and 97 % of BCC infections in CF patients. Conversely, the potential for this pathogen to infect non-CF patients seems limited, although its epidemiology is poorly understood. The 'cepacia syndrome,' which consists of fever, fast advancing necrotizing pneumonia and bloodstream infection, has been linked to BCC species, driving to severe lung decline and increased mortality in CF individuals, although it has been also described in very rare cases of non-CF patients (Hauser & Orsini, 2015; Spencer et al., 2020).

Achromobacter xylosoxidans (AX) is a Gram-negative, mobile, oxidase and catalase positive, non-fermenting rod-shaped bacterium, and similar to BCC species, displays a limited capacity to cause acute infections. However, its role as cause of chronic infection in CF individuals is not despicable at all. In this sense, when compared to control CF patients who are not infected with this bacterium, AX presence is associated to a greater deterioration in respiratory function and frequency of exacerbations. It has also been demonstrated to drive inflammation in CF patients at a degree comparable to that brought on by PA (Menetrey et al., 2021).

The question that arises here is why these species of non-fermenting Gram-negatives are not as successful as PA for causing chronic infection in CF patients, as can be observed in Figure 1. The explanation is probably multi-factorial, and likely, the greater metabolic versatility, adaptability and capacity for antibiotic resistance development of PA strongly support its outstanding prevalence. However, the inter-species competition mechanisms that appear in natural environments but also in the particular CF niche probably have also a great impact on this topic, as will be shown in the next section (Rossi et al., 2021).

5. *Pseudomonas aeruginosa*: mechanisms of competition

It is widely recognized how important the human microbiome is to overall health, and numerous studies have shown that the dysbiosis of the typical microbial populations in any given anatomical region is directly linked to the development of diseases. A diverse community of microbes makes up a healthy microbiome. These communities are much more resilient to pathogenic species blooms and therefore have beneficial effects for the host. By secreting antimicrobial peptides and other compounds to inhibit the growth of

pathogens, commensal bacteria maintain community homeostasis. However, the diversity is significantly smaller in dysbiotic communities linked to chronic disease and is frequently dominated by a small number of pathogenic species, as for example PA in the CF niche (Welp & Bomberger, 2020). In this regard, PA has numerous strategies and related features for competing and occasionally even co-existing with other pathogenic bacteria, which end up influencing the outcomes of infection. Some of these phenomena of co-existence/competition are:

Polymicrobial Biofilm Formation. Pathogens encounter a variety of different species in the respiratory system, which may change how bacterial communities are organized. For instance, co-infection with other species has been found to alter the way PA builds biofilms. Additionally, bacterial species can influence nearby bacteria to promote the creation of biofilms, enhancing both species' tolerance and persistence. For instance, PA protects other nearby species by producing the so-called extrapolymeric substance (EPS), which is essentially the biofilm matrix, made of the abovementioned polysaccharides, Psl, Pel, and mainly alginate. There is evidence that each of the three polysaccharides makes other bacteria (that do not produce such robust biofilms) more resistant to antibiotics, which could help to increase the diversity of species within the biofilm, even driving to polymicrobial infection episodes (Colvin et al., 2012; Welp & Bomberger, 2020). This has been shown for instance in mixed biofilms of PA and the Gram-positive *S. aureus*, in which chronic adaptation apparently promotes eventual co-existing interactions between them in the CF context (Camus et al., 2021).

Exploitative competition. All bacteria in the respiratory tract require some basic nutrients and substrates in order to survive. Bacteria must either seize these nutrients for themselves or get beyond the mechanisms that other microbes use to get these restricted resources in order to ensure access to them (Figure 2). These competing behaviors, closely related to the metabolic versatility and biological fitness of each species, play a crucial role in the ecology and diversity of the respiratory microbiome, which has an impact on chronic respiratory disease (Hood & Skaar, 2012). The bacterial mechanisms to reach and occupy a physical space within a niche are also considered a part of this type of exploitative competition.

Growth interference. Certain enzymatic and non-enzymatic chemicals that bacteria produce and release to the extracellular medium can either kill or obstruct microbial growth (Figure 2). Pyocyanin, just one example of the different PA metabolites with

antibacterial activity, has been specifically shown to diminish the variety of microbial communities because it prevents many other species from growing, including certain species of BCC, allowing PA to outcompete them in polymicrobial biofilms (Malhotra et al., 2019). Some of these compounds causing growth interference, for which the direct contact between cells is not needed, can be released directly to the medium, or conversely, within small fragments of outer membrane, called Outer Membrane Vesicles (OMVs), that exert their effects once fused with the envelopes of the target cells.

Contact – Dependent Toxin delivery. Although bacteria have a variety of strategies for competing with nearby organisms in their immediate environment, some of these strategies need closer contacts with nearby cells. As they depend on direct physical interaction to move toxins and other chemicals from cell to cell, these systems are contact-dependent. One such method is the contact dependent inhibition (CDI), which is utilized to compete with neighboring cells (Figure 2). Species harboring CDI systems form a rigid extracellular filament topped with a receptor binding domain which links to its cognate outer membrane receptors on target bacteria, triggering different mechanisms of cell death. There are further contact-dependent growth inhibition mechanisms, such as the type VI secretion system (T6SS), described to work in a syringe-like mode of action, inoculating effectors in the prey cell, that can be either from the host, or a bacterial competitor (Welp & Bomberger, 2020). In the next section, the main features of this efficient competition system are approached.
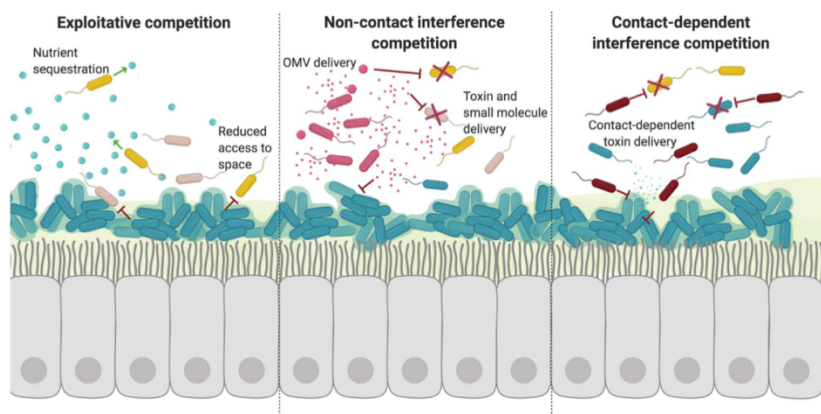

Figure 2. Microbial communities are modulated by different competitive relations (Welp & Bomberger, 2020).

5.1 The type VI Secretion System (T6SS)

The T6SS and certain bacteriophage proteins have a shared evolutionary history (Leiman et al., 2009). These "molecular needles" are contractile, contact-dependent structures that pierce cell membranes to deliver harmful effectors into neighboring cells, and that are not constitutively expressed in PA, but follow a dynamic of assembly-disassembly depending on the circumstances, the so-called "tit for tat" model (Figure 3).

Numerous toxic effectors delivered by T6SS have been found to cause cell death or growth suppression, through different mechanisms, including membrane permeabilization, nucleic acid degradation, peptidoglycan breakdown, and transcriptional/translational inhibition. PA is one of the species with a more deeply studied T6SS, consisting of various non-redundant T6SS machineries that work to deliver a variety of toxins to nearby cells. In fact, PA has three loci codifying for three co-existing homologue T6SS, namely H1, H2 and H3, each of them with their own specific features, compounds, specialized targets (host *vs*. bacterial cells) and particular effectors. Some of the most studied PA T6SS effectors targeting bacterial competitors are Tse1, Tse2, Tse3, Tse5, PldA, VgrG2b, Tle3, Tle4, etc. These effectors always work in a toxin-antitoxin, fashion; that is to say, for instance while the effectors Tse1 and Tse3 hydrolyze peptidoglycan of target cells, PA utilizes their periplasmic immunity proteins Tsi1 and Tsi3, in order to mitigate the toxicity of Tse1 and Tse3, preventing itself from being killed by sister cells (Sana et al., 2016). A general scheme of the main components of the PA T6SS and their mechanism of action (using the specific example of Tse1 injection) is shown in Figure 3.

In any case, it has been seen that even though the T6SS functions effectively *in vivo*, the system is heavily repressed *in vitro*, usually requiring the use of a RetS-deficient background in order to de-repress the T6SS. This is so, because RetS is a multifunctional regulator that controls a variety of virulence characteristics related to acute infection and chronic persistence in PA, including the T3/6SS and the development of biofilm. In fact, it is quite typical the selection of PA *retS*-defective mutants in the CF context, which are characterized by a down-regulation in the T3SS expression, and an over-activation of T6SS and alginate production (Miller, 2013; Chen et al., 2015; Welp & Bomberger, 2020). Thus, the over-activation of the PA T6SS in this context poses a paradigm for interbacterial competitiveness based on defensive counterattack with precise timing and location (Figure 3; Miller, 2013). Moreover, to study this system in the laboratory, it is required to assess it in solid media to conduct competitions since, in a liquid environment, bacteria apparently cannot establish interactions robust enough to form the injection systems.

Finally, it has been recently shown that in addition to the effectors for the T6SS previously documented, such as Tse, Tle, etc., one of the starring actors of this work, namely AmpDh3, appears to function as an effector protein in this system, as will be shown in the next section (Wang et al., 2020).
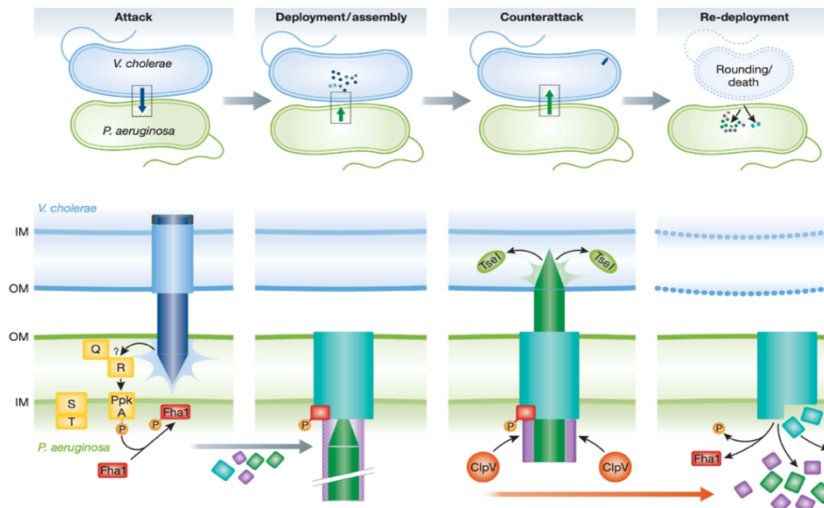
Figure 3. An example of PA T6SS targeting *Vibrio cholerae*. The PA TagQRST signal-transduction system (yellow) detects T6SS assault from *V. cholerae* and causes the PpkA kinase to become activated and phosphorylate Fha1. Phosphorylated Fha1 controls the assembly of a T6SS system close to the place of assault by firing the inner tube/spike complex (dark green) upon contraction of the outer sheath (violet). The target *V. cholerae* is partially destroyed by effector proteins supplied by the T6SS, such as the Tse1 peptidoglycan amidase. The ClpVATPase and Fha1 dephosphorylation assist the disassembly of the fired apparatus to enable redeployment to new places (Miller, 2013). This mode of action of PA T6SS only assembling in response to a previous aggression is commonly known as "tit for tat".

5.2 The role of AmpDh3 in *Pseudomonas aeruginosa*

It was classically considered from more than a decade ago that the PA AmpDh3 amidase was involved in the regular process of peptidoglycan turnover and recycling, also being linked to the intrinsic AmpC β–lactamase regulation as a kind of indirect repressor of its production. In fact, it was shown that upon the inactivation of this amidase, the development of different levels of β-lactamase expression and β-lactam resistance, were achieved in combined mutants lacking the *ampDh3* gene together with its *ampD* or *ampD-ampDh2* amidase homologues. (Juan et al., 2006; Moya et al., 2008). It was believed that besides this indirect AmpC-repressor role, the natural function of AmpDh3 was to cleave and release stem lateral peptides from the glycan chains in the peptidoglycan sacculus, to initiate its regular remodeling and turnover. It is important to state that the peptidoglycan is not a static structure, but a very dynamic element, that needs to be continuously degraded/re-built, to allow cell growth, division and other processes, for which periplasmic amidases are thought to be essential. In fact, it was shown *in vitro* that the substrate of AmpDh3 was the entire peptidoglycan sacculus rather than soluble fragments, and therefore it was hypothesized that this amidase was

periplasmic, opposed to the cytosolic AmpD homologue, specialized in cleaving stem peptides from soluble peptidoglycan pieces obtained from peptidoglycan-controlled remodeling (Zhang et al., 2013; Juan et al., 2017; 2018).

However, more recently it has been proposed that the actual role of PA AmpDh3 is not to degrade the peptidoglycan of the producer cell, but to be injected into the periplasm of rival cells via T6SS (specifically through H2-T6SS), to degrade their peptidoglycans (Figure 4), acting as a cell-wall amidase effector to increase PA competitiveness. In fact, it has been established that AmpDh3 is crucial for the T6SS-mediated competition of PA against *Yersinia pseudotuberculosis* and *Escherichia coli*. It has been also shown in this context that PA produces the auto-immunity protein (anti-toxin) PA0808 which avoids the effects of AmpDh3 within the sister cells (Wang et al., 2020). Still, since PA plays a significant role in CF but neither *Y. pseudotuberculosis* nor *E. coli* have an impact on CF, we were particularly interested in studying the role that AmpDh3 may play in the competition with other microorganisms that can coexist/compete with PA in the lung of CF patients, such as the abovementioned SM, BM and AX, a topic never approached before.
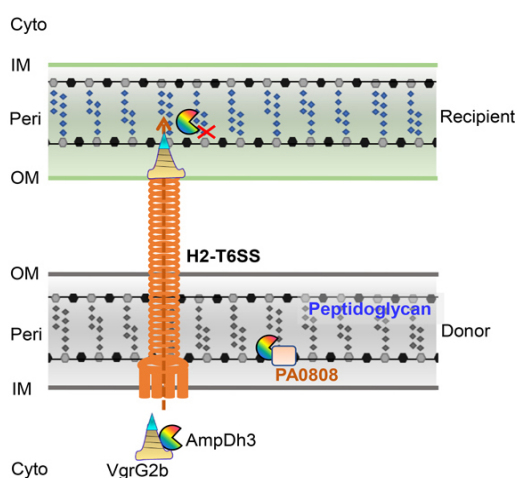

Figure 4. Delivery model of AmpDh3 to the competing cells' periplasm by PA H2-T6SS. The auto-immunity protein PA0808 and AmpDh3 are shown as rectangle and "packman," respectively. It is also shown that AmpDh3 interacts with the protein VgrG2b into the cytosol of PA, a step required for its effective secretion (Wang et al., 2020).

ANTECEDENTS, HYPOTHESIS AND OBJECTIVES

The alarming clinical-epidemiological threat posed by the increasing antibiotic resistance levels acquired by PA, especially in hospital environments and in the particular context of CF, makes necessary the search for new antipseudomonal treatments. However, a first necessary step to develop them is to identify therapeutic targets. In this regard, an interesting option could be to fully understand how PA manages to be such a good competitor in the CF niche. In other words, dissecting the role that certain poorly characterized elements allegedly important for competition such as AmpDh3, could

enable them as interesting weak points to be attacked. Thus, if this or other toxins delivered by the T6SS were found to be indispensable for the competition in the CF lung, a therapy intended to disable these competitiveness-related weapons could disarm PA against other microbes. This strategy could therefore deviate the dynamics of colonization of CF patients towards less dangerous species displaying lower virulence/antibiotic resistance, which could increase our probabilities of therapeutic success against them.

Leaning on these bases, we hypothesized that the T6SS system in general and the AmpDh3 toxin in particular, could be indispensable weapons for PA to compete in the CF niche against other notably important gram-negative non-fermenters such as SM, BM and AX. This would be supported by the already demonstrated importance of these elements to compete against other microbes not linked to chronic infection (*Y. pseudotuberculosis* and *E. coli*). To ascertain this hypothesis, the general goal of our work was: to analyze the role that the T6SS in general and the AmpDh3 amidase in particular play in the PA competition capacity against different species of gram-negative non-fermenters such as SM, BM and AX, known to have a notable relevance in the CF-associated chronic infection, topic never approached before.

To achieve this general goal, the following specific objectives were drawn:

1. To construct a PA *ampDh3* knockout mutant using a *retS*-deficient strain (that shows a constitutive T6SS activation) as a template and as a comparator and study these mutants in parallel to the original wildtype PAO1 and its previously obtained *ampDh3*–defective strain.

2. To analyze the potential differences in the growth rate caused by the mentioned gene inactivation in PA, but also potentially existing among reference strains of SM, BM and AX, when inoculated in solid medium.

3. Analyze the competition capacity of the abovementioned PA strains when inoculated in competition experiments (solid media) with the Gram-negative non-fermenters SM, BM and AX.

MATERIALS AND METHODS

1. Plasmids and bacterial strains used

Table 1. Bacterial strains and plasmids used in this master's thesis.

STRAIN	GENOTYPE/IMPORTANT FEATURES	REFERENCE

17

PAO1	*P. aeruginosa* reference strain with fully sequenced genome, considered invasive.	Stover et al., 2000.
PAΔADh3	PAO1 Δ*ampDh3::Gm*. *ampDh3* encodes the AmpDh3 amidase, a toxin targeting the peptidoglycan of competitor bacteria and delivered through T6SS.	Juan et al., 2006.
PAΔretS	PAO1 Δ*retS::ISphoA/hah-tc* *retS* encodes a master regulator of virulence-related features. Its disruption drives to constitutive activation of T6SS. Mutant obtained from a transposon mutant library (University of Washington)	Jacobs et al., 2003.
PAΔretSΔADh3	PAO1 Δ*retS:: ISphoA/hah-tc* Δ*ampDh3::Gm*. *ampDh3*-defective mutant constructed on the PAΔretS strain as template, using the below described cre-lox protocol.	This work.
S. maltophilia **LMG 958**	Type/reference strain of *S. maltophilia.*	Carmody et al., 2011.
A. xylosoxidans **LMG 1863**	Type/reference strain of *A. xylosoxidans.*	Yabuuchi & Ohyama, 1971.
B. multivorans **LMG 16775**	Type/reference strain of *B. multivorans.*	Baillie et al., 2013.
E. coli S17-1	Laboratory strain used for conjugation experiments needed for *ampDh3* inactivation.	Simon et al., 1983.
PLASMIDS		
pEXTADh3Gm	Construct harboring the *ampDh3* gene disrupted by a gentamicin resistance cassette, (based on pEX100Tlink gene replacement vector). Used for *ampDh3* inactivation following described protocols.	Quénée et al., 2005; Juan et al., 2006.

Abbreviations: *ISphoA/hah-tc*: transposon disrupting the *retS* gene in the PAΔretS strain; Gm: gentamicin resistance cassette flanked by lox sequences, used for cre-lox inactivation protocol (Quénée et al., 2005).

2. Mutant construction protocol

The cre-lox gene inactivation system using the previously constructed plasmid pEXTADh3Gm was followed to inactivate *ampDh3* in the PAΔretS strain (Quénée et al., 2005; Juan et al., 2006).

Briefly, the PAΔretS and *E. coli* S17-1 harboring pEXTADh3Gm were conjugated to allow the transfer of this plasmid to the PA strain, enabling homologous recombination and the consequent replacement of wildtype *ampDh3* gene by the gentamicin resistance cassette-disrupted one. To perform the conjugation, these steps were followed:

1) Overnight cultures (5 ml) of the strains to be conjugated were prepared in LB (Luria-Bertani) broth (Annex I; page 37) and incubated at 37ºC and agitation (180 rpm). Gentamicin at 5 µg/ml was added to the culture of *E. coli* S17-1 harboring pEXTADh3Gm, to avoid the loss of the plasmid.

2) The day after, the cultures were diluted 1:40 in fresh LB broth and incubated for 2-3 hours at 37ºC and 180 rpm until they reached the exponential phase, i.e., an optical density at 600 nm (OD_{600}) of ≈0.5 (approx. 5×10^8 bacteria/ml).

3) The strains to be conjugated were mixed in a new tube: 500 µl of exponentially growing PAΔretS were added to 5 ml of exponentially growing S17-1 pEXTADh3Gm, and the suspension was centrifuged 15 min at 2000 x g.

4) The supernatant was removed, the pellet was resuspended in the remaining LB of the tubes (about 100 µl) and the suspension was spread in the form of a button in the center of a commercial blood agar plate (Biomérieux).

5) The button was incubated 6 hours at 37ºC, resuspended in 1000 µl of sterile saline solution (0.9% NaCl) and then plated (200 µl/plate) on Müller-Hinton (MH) agar (Annex I; page 37) supplemented with sucrose (5%), gentamicin (30 µg/ml), and cefotaxime (1 µg/ml, concentration sufficient to kill *E. coli*, but not affecting PA).

6) Appropriate negative controls (buttons made of PAΔretS alone or S17-1 pEXTADh3Gm alone) were also prepared following the same protocol, and finally plated in the same type of MH agar plates.

7) All the plates were incubated overnight at 37º C.

The only colonies growing in the abovementioned plates should be the double recombinants, in which *ampDh3* is replaced by the *ampDh3*::Gm variant proceeding from pEXTADh3Gm. This double homologous recombination is the unique option for survival, since it entails the loss of the rest of the plasmid, due to the pEX100Tlink plasmids

incapacity to replicate in *Pseudomonas* (suicide vector), the requirement to integrate the gentamicin resistance cassette to survive in the plates, and the requirement for losing the rest of the vector, that encodes the *sacB* gene (lethality induced by sucrose). To check that the candidate colonies were correct, these steps were followed:

8) Sequential passages of the candidate colonies on MH agar plates supplemented with sucrose (5%), gentamicin (30 g/mL), and carbenicillin (200 g/mL) followed by passages (using the same colony/loop) in plates with the same components but without carbenicillin were carried out. The double recombinants cannot grow in the first type of plates, as they no longer have the *bla* gene that confers resistance to carbenicillin, but they should grow in the second type, since they also lack *sacB* (and actually the rest of the pEXTADh3Gm suicide vector), but still harbor the gentamicin resistance cassette.

9) Final confirmation of the double recombinants was carried out by PCR amplification using specific primers for *ampDh3* (Table 2), the appropriate reagents mixed as appear in Table 3, and the conditions: 94°C, 10 min + [94°C, 1 min / annealing temperature 59 °C, 1 min / 72°C, 1 min] x 35 cycles + 72 °C, 10 min (using a BIO-RAD PTC-100 thermal-cycler device). The DNA of each strain was obtained through the High Pure PCR template preparation kit (Roche), following the manufacturer instructions.

10) Finally, 0.5x TBE (Annex I; page 37) and a mid-resolution agarose 1% gel (Ecogen) was made to verify the PCR results.

Table 2. Primers used in the PCR.

PRIMER	SEQUENCE (5-3')	ANNEALING TEMPERATURE (ºC)	AMPLIFIED GENE	PCR PRODUCT SIZE, bp
AD3-F	TTGGCCGGCCCCTGAAC	59	*ampDh3*	768
AD3-R	GCGACGACCTGAGCGACG			

Table 3. Reagent mixture for PCR (25 µL final volume, 1 sample).

REAGENTS	VOLUME PER REACTION* (µl)
dH$_2$O	17.25
Buffer 10x	2.5
Dimethyl sulfoxide (DMSO)	2.5
MgCl$_2$ (25 mM)	1.5

dNTPs (10 mM each)	0.5
Primer F	0.25
Primer R	0.25
Taq polymerase (AmpliTaq Gold^{TM})	0.25
DNA template	1

* It should be multiplied by as many samples as the investigator wants to make, in this concrete work, 10 reactions.

3. Bacterial competition protocol

Considering that a minimum of three independent replicates of each PA strain (PAO1, PAΔADh3, PAΔretS, PAΔretSΔADh3) *vs*. each competitor species (SM, BM and AX) were done, LB broth overnight cultures (10 mL) were routinely prepared the day before the experiments, in 50 ml tubes with the planned bacterial strains. The day after, the overnight cultures were centrifuged at 2000 x g for 10' at 4ºC. The supernatant was then removed, and 10 ml of Dulbeccos Phosphate Buffered Saline (PBS, Biowest) were added to wash the cells and eliminate released substances potentially affecting the competition assays. A second round of centrifugation was conducted in all the cases, with a final resuspension in 10 ml of PBS.

The tubes were put on ice to prevent bacterial growth after the second centrifugation, and the optical density at 600 nm (OD$_{600}$) was assessed using a spectrophotometer Shimadzu UV-1280.

Preliminary experiments to determine the equivalences between the OD$_{600}$ values and bacterial numbers were performed, using appropriate serial dilutions, platings and colony counts. Finally, the correspondence of OD$_{600}$=1 with $1E^9$ colony forming units (CFU)/ml of suspension was stablished for all the species, except for SM, for which this OD value accounted for $1E^8$ CFU/ml.

Once stablished these parameters, the competition assays were carried out following previously described protocols (Perault et al. 2020; Nas et al. 2021) with minor modifications, always with a proportion of 1:1 between each PA strain and its competitor. Briefly, the desired initial inoculum of each species in competition was $1E^6$ CFU in all the cases, and the dynamics for samples' preparation and assays performance were as follows:

3.1 Bacterial inoculums at time 0h

Following the abovementioned correspondences between OD_{600} and CFU/ml, and making the appropriate intermediate dilutions with PBS, a final suspension containing $1E^6$ CFU of each competitor in a final volume of 50 µl completed with PBS was prepared in a regular PCR tube (separately for each bacterium). This suspension was mixed by pipetting, and different serial dilutions were plated in the appropriate agar plates (see Table 4). After 24h of incubation at 37ºC, a colony count was carried out to verify that a correct inoculum of $1E^6$ CFU of each competitor was prepared at time 0 (a deviation of up to 25% of the desired inoculum was considered as acceptable). <u>Exception</u>: given the slow growth of AX, its colonies were counted after 48 h.

3.2 Competition buttons (Nº of viable bacteria after 4 h)

Parallel duplicates of the suspensions explained in the previous paragraph were exactly prepared for competition assays, but in this case, $1E^6$ CFU of both competing bacteria and the corresponding PA strain were combined and PBS was additionally added to achieve the desired final volume of 50 µl. Afterwards the entire 50 µl suspension was placed in the center of a blood agar plate, in the form of a button.

Then, the plates were incubated at 37°C for 4 h. After these 4 h, all the bacterial mass was carefully collected with a sterile plastic loop and placed in an *Eppendorf* tube containing 500 µl of saline solution. Subsequently, the suspension was thoroughly mixed to make different serial dilutions, and depending on each strain's characteristics (Table 4), different agar plates were used for plating and colony counts. Briefly, when clear morphological differences existed between the two competitors, platings at time 0h and 4h were carried out in regular MH agar plates and the following day, the respective colony counts enabled to determine the initial and final numbers of each species. Conversely, when the two competitor strains were not easily differentiable by their appearance, an antibiotic marker had to be used, and a parallel plating in regular MH plates *vs.* MH supplemented with the appropriate antibiotic allowed to determine the total number of bacteria *vs.* the number of CFU of the resistant strain, respectively. Obviously, each competitor was easily quantified just subtracting the latter from the former number, both at time 0h and 4h.

Table 4. Differences in bacterial competition assays.

Competition	Method used to differentiate competitors	Notes

P. aeruginosa vs. *S. maltophilia*	<u>*P. aeruginosa*</u>: growth only in MH plates with no antibiotic. <u>*S. maltophilia*</u>: growth also on MH plates supplemented with imipenem 10 mg/l.	*S. maltophilia* is naturally resistant to imipenem.
P. aeruginosa vs. *B. multivorans*	No antibiotics used: clear visual differentiation between species.	<u>*B. multivorans*</u>: small opaque colonies, slightly yellowish. <u>*P. aeruginosa*</u>: larger, rounded, whitish colonies.
P. aeruginosa vs. *A. xylosoxidans*	<u>*P. aeruginosa*</u>: growth only in MH plates with no antibiotic. <u>*A. xylosoxidans*</u>: growth also on MH plates supplemented with ciprofloxacin 5 mg/l.	*A. xylosoxidans* is naturally resistant to ciprofloxacin.

Finally, by considering the colony counts and the plated dilutions, the numbers of CFU of each competitor present in the initial inoculum and in the button after the 4 hours, were easily calculated. Subsequently, a Competition Index (CI) was determined following previously described protocols (Perault et al., 2020) and using the formula:

$$(\text{PA population}_{T4h}/\text{competitor population}_{T4h})/(\text{PA population}_{T0}/\text{competitor population}_{T0})$$

Although the initial inoculums were accurately adjusted to have the highest degree of similarity ($1E^6$ CFU), as can be observed this formula enabled a normalization to minimize the effect of potential initial differences. Obviously, when CI was above 1, it meant that PA strain outperformed the competitor species; when CI was below 1, it meant that PA strain was outcompeted by the competitor, and if CI was around 1, it meant that both species had a similar competition capacity (Perault et al., 2020).

4. Single-bacterial species growth protocol

The same procedure explained in section 3.2 was followed, but instead of combining two species in a single competition suspension/button, each bacterial strain was seeded independently. Serial dilutions and platings in regular MH plates at time 0 and 4h were carried out to enable colony counts after overnight incubations at 37ºC (48h in the case of AX). The division of the calculated final by initial numbers of bacteria allowed establishing a population growth factor to make easier the interpretation of data (see results). At least three independent replicates of each species or PA strains were carried out.

5. Statistical analysis

A mean and standard deviation were calculated from all the values of the examined parameters (CI, population growth factor) that were separately acquired for each experimental replicate, to enable statistical comparisons between the strains. The *GraphPad Prism 7* software was then used to conduct a statistical analysis to establish whether or not the differences in the mean values for each strain/parameter were significant. To do this, an ANOVA test and a Tukey post-hoc test for multiple comparisons were used to evaluate the data. Accordingly, a p-value of 0.05 or below was regarded as statistically significant. The graphic representations of the research outcomes were also created using the *GraphPad Prism 7*.

RESULTS

1. Construction and testing of *ampDh3*-deficient mutants

To verify that the construction of PAΔretSΔADh3 mutant was successful, the candidate colonies (growing in sucrose plus gentamicin plates but not in carbenicillin ones) obtained after the assays explained in Methods section were checked through a PCR of the *ampDh3* gene with the abovementioned primers AD3-F and AD3-R. The results of this PCR were run on a 1% agarose gel (Figure 5), and the size of amplicons indicated that the process was correct: the positive control PAO1 (wild type), provided a band of a molecular weight of ca. 750 bp (*ampDh3* has a size of 768 nucleotides), clearly different from the size of the constructed mutants, which displayed a molecular weight of ca. 1500 pb (the gentamicin resistance cassette interrupting *ampDh3* was the responsible for this size increase). The previously constructed mutant PAΔADh3 (Juan et al., 2006) was also included as a control of the size of *ampDh3* interrupted by the gentamicin resistance cassette.

Figure 5. The gel shows the result of the PCR performed to check the PAΔretSΔADh3 double mutant. The samples loaded on the wells are: 1-PAO1; 2 - PAΔADh3; 3, 4, 5, 6, 7, 8, 9 – candidate colonies of PAΔretSΔADh3; 10 – negative control.
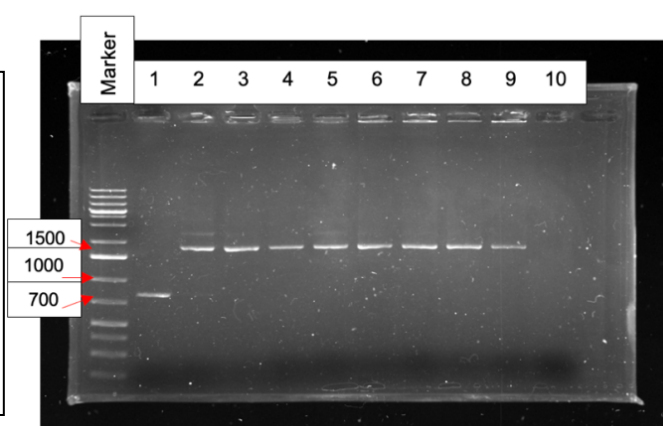
2. Determination of the plate growth rate of the different species/strains used

The results regarding the growth in solitary of each of the strains/species in solid medium (mean value of the obtained population growth factors), shown in Figure 6, revealed that there are significant differences (p-value ≤ 0.05) for the growth (4 h) of SM, with higher values (population increases of ca. 40-fold with regards to the initial inoculum) compared to the other species (population increases ca. 20-fold or less for the rest of strains/species). In addition, significant differences were also found in the PA strains, with those displaying a wildtype *retS*, (PAO1 and PAΔADh3), having a greater growth (ca. two-fold) compared to PAΔretS and PAΔretSΔADh3. Interestingly, BM and especially AX were the species with lower growth in the used conditions.
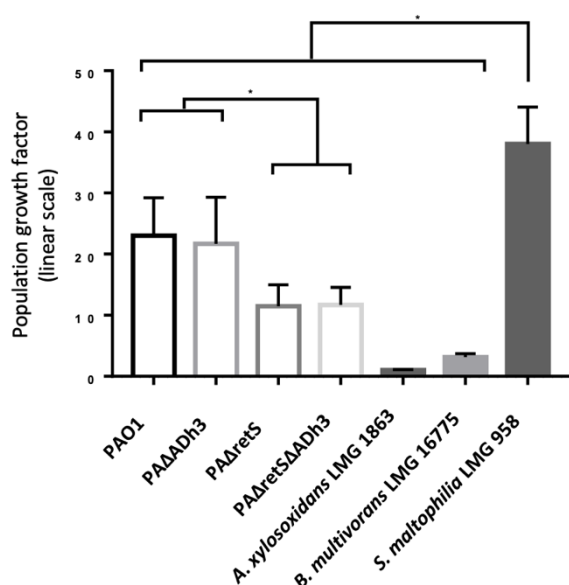

Figure 6. *P. aeruginosa* strains and competitor species average growth in solid medium. The population growth factor stands for the fold-increase in the number of bacteria at 4h with regards to the initial inoculum. The columns represent the mean values of the calculated factors of all the experimental replicates, whereas the error bars are the standard deviations. The asterisks indicate statistically significant differences between strains and species.

3. Determination of the competition index (CI) of the different strains used

Figure 7 shows the competition index (CI) of the different PA strains against SM. As stated in Methods section, and as can be seen in the figure, the CI value clearly below 1, indicates that SM outcompeted the strains of PA with an active *retS* (which entails a repressed T6SS, p-value ≤ 0.05). This was not the case for the *retS*-defective strains, (entailing a constitutively functional T6SS), in which a CI greater than 1 was observed, thus PA being more competent than its rival SM. Interestingly, PAΔretSΔADh3 had a CI closer to 1 compared to PAΔretS (although not reaching statistical significance), suggesting some role of AmpDh3 for the competition *vs*. SM when the T6SS is active.

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Figure 7. CI for the competitions between PA strains and SM. The columns represent the mean values of the CI of all the experimental replicates, whereas the error bars are the standard deviations. The asterisks indicate statistically significant differences between strains/species.

Figure 8. CI for the competitions between PA strains and AX. The columns represent the mean values of the CI of all the experimental replicates, whereas the error bars are the standard deviations. The asterisks indicate statistically significant differences between strains/species.
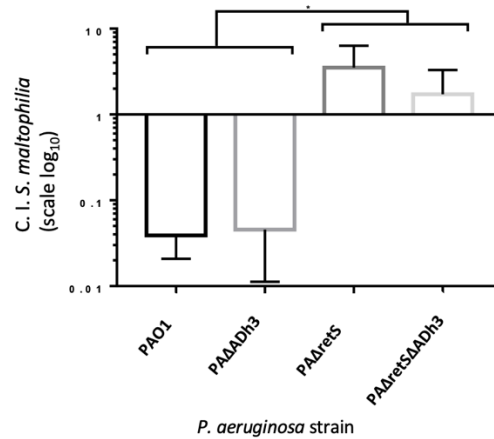

Figure 9. CI for the competitions between PA strains and BM. The columns represent the mean values of the CI of all the experimental replicates, whereas the error bars are the standard deviations. The asterisks indicate statistically significant differences between strains/species.
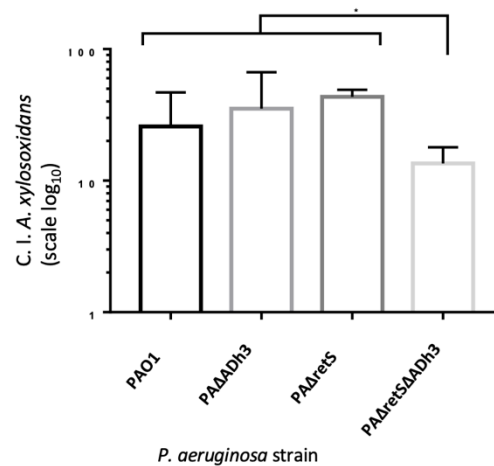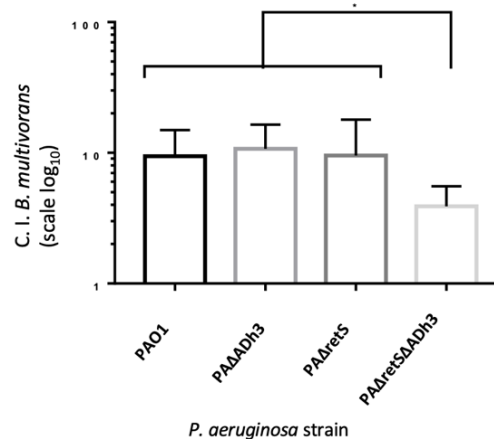
In Figure 8, a CI much higher than 1 can be observed (ca. 25), for PAO1 competing strongly better against AX, and a trend even for a better competitiveness (although not reaching statistical significance) could be observed for PAΔretS (CI ca. 40), which was expectable given the activation of T6SS. Interestingly, when *ampDh3* was disrupted in this latter background, the competitiveness of PA was significantly reduced (CI ca. 10).

The results of competition between PA and BM are depicted in Figure 9, with a CI of ca. 10 for PAO1, PAΔretS and PAΔADh3 indicating that PA outcompeted its rival. Additionally, a statistically significant difference between the CI of PAΔretSΔADh3 and those of the other PA strains was also seen (reduced at ca. 50%), in a similar way as happened for the AX competition, and suggesting some role of AmpDh3 for the PA competitiveness against these species when T6SS is active.

DISCUSSION

Regarding the role of T6SS in the competitiveness of PA against different gram-negative non-fermenters in the CF niche, a first intriguing result obtained through this work is the fact that, in the competition between BM and PA, the inactivation of *retS* did not affect *per se* the outcomes (Figure 9). This would suggest that the T6SS is not important at all for the competitiveness of PA against BM. In contrast, the *retS*-defective PA strain competed much better than PAO1 *vs.* SM, with a CI that was roughly 100 times higher, indicating the essential role that the activation of T6SS plays in the competitiveness of PA against SM. In fact, SM clearly outcompeted PA when this species had a repressed T6SS (because of an active *retS*). However, despite these *in vitro* results, it has also been demonstrated in other papers that under *in vivo* circumstances, SM and PA are capable of cooperating and not confronting each other (McDaniel et al., 2020), which reveals the complexity of the interactions that these two species may stablish in the CF niche. In the case of the competitions with AX, the activation of T6SS apparently had some effect increasing the PA competitiveness (although not reaching statistical significance in the comparison between PAO1 and PAΔretS, p-value > 0.05). This would suggest that the T6SS activation *per se* plays some modest role increasing the competitiveness of PA against AX, but with much less importance than in the competition against SM. However, it is important to keep in mind that *in vitro* models do not have to be exactly equivalent to what happens in an infection, but they are a valuable first experimental approximation.

It is also important to remember that the T6SS is barely expressed *in vitro* whereas it is highly induced during infections, to improve competitiveness between species. Thus, to mimic this situation, the defective background in *retS* has been repeatedly used, because this general regulator, when inactivated, de-represses the expression of T6SS (Lien & Lai, 2017; Wang et al., 2020). Moreover, the assays to assess the role of T6SS have been performed on plates because it requires stable cell-cell contact, which is unlikely to happen if the cells are in planktonic state (liquid medium). Due to the thick peptidoglycan

of Gram-positive bacteria, the T6SS is believed to be ineffective against them, but has been repeatedly highlighted as a very important competition weapon of PA against different gram-negatives. However, the role of T6SS against certain Gram-negative species thought to be competitors of PA for the CF niche, such as SM, AX and BM has been barely studied before, which justifies our work (Hood & Skaar, 2012; Wang et al., 2020). Our results regarding the differential importance that PA T6SS apparently plays in the competition depending on the rival species could be likely supported, at least partially by the fact that PA T6SS functions in accordance with the *tit for tat* model, in which PA T6SS does not function unless PA is previously attacked by another species that has also has a working T6SS. Therefore, even if *retS* has been deactivated, PA apparently does not effectively use its T6SS to target a bacterium that lacks this particular mechanism, and that consequently, has not previously attacked PA. The method that different bacteria use to assemble T6SS must also be considered, because for instance, PA only activates this assembly in the specific point in which it has been attacked by the aggressor species' T6SS. Meanwhile, some other bacterial species, such as *Vibrio cholerae* or *Serratia marcescens*, act in a more haphazard manner (though a random assembly of T6SS) and release toxins without selecting a specific point of attack, raising the possibility that these variations trigger different PA responses (Basler et al., 2013; Smith et al., 2020). Our data support the prominent role that T6SS plays, when activated, in the competition of PA against SM, but not against BM. Although SM and BM both express a T6SS, (Nas et al., 2021), maybe BM uses a T6SS differently than PA or SM does, since it's not quite sure how BM's model works, if *tit for tat* or *random*. Therefore, one scenario could be that even in a *retS*–deficient background, and in a situation in which the competitor also possesses T6SS, some particular ways of T6SS assembly (as could be the case of BM) could prevent the T6SS activation of PA. Future work will be needed to fully understand how the BM T6SS works.

Another interesting aspect of the topic is that, despite the evidence of the coexistence of both species and some other interactions such as adhesion or biofilm formation (Sandri et al., 2021), there are currently no studies that compare the competitiveness of PA and AX in solid medium. On the other hand, apparently, AX lacks a T6SS, which implies that it cannot attack PA, and therefore, theoretically speaking, AX should not enable the tit for tat-mediated activation of T6SS in PA. However, and although it was not statistically significant, we observed an intriguing trend of the *retS*-defective PA to compete better against AX. This could be either due to an experimental artifact, or because PA somehow displays a small activation response of its T6SS, specifically against AX, linked to the

retS inactivation without the need for a previous T6SS aggression. More experiments in this direction will be needed to ascertain these hypotheses.

The differences existing between PA and the other species studied in this work regarding their solitary growth in plates, although very important quantitatively speaking, are not a decisive issue for the outcomes of our competitions. That is to say, once stablished the differences in the basal growth, the use of KO mutants was intended to reveal even greater differences or significant reductions in the growth of the competing strains, to determine the importance of T6SS and/or AmpDh3. In fact, we observed an impaired growth of the *retS* –defective strains, that was otherwise compensated by a better competition capacity for instance against SM, indicating again the minor influence of the solitary growth rates.

Regarding the role that AmpDh3 may play for the competition of PA against SM, BM and AX, there were no changes between the PAΔADh3 CI and those of PAO1 when tested against any of the competitor species. This was expectable, since, if T6SS is not active, the presence/absence of one of its toxins (AmpDh3) should have no effect, a circumstance that our results demonstrate. Contrarily, it appears that the double mutant PAΔretSΔADh3 competed worse against the three competing bacterial species AX, SM, and BM, than the PΔretS strain. This would demonstrate that in a situation in which the T6SS is working, the delivery of AmpDh3 toxin contributes to the competitiveness of PA against SM, BM and AX, although with a quite modest impact.

Intriguingly, our data show that PAO1, PAΔADh3, and PAΔretS strains behave very similarly against BM, whereas PAΔretSΔADh3 significantly lost competitiveness. It would be more expectable that the PAΔretS strain competed better than wildtype PAO1 because of the derived T6SS activation, but this was not observed in our results. One explanation for this finding could be the need for more replicates of our experiments, that could finally reveal an increased capacity for competition in PAΔretS compared to PAO1, that would be lost in PAΔretSΔADh3. In addition, it should be noted that some degree of controversy exists, because although the AmpDh3 amidase is secreted by the H2-T6SS and has been shown to be essential for PAO1 strain competition against non-CF related gram-negatives (Wang et al., 2020) other works defend that, from the three distinct T6SS that PA produces, the H2-T6SS and the H3-T6SS clusters are by far less effective in killing bacteria than the H1-T6SS (much more important for the inter-bacterial competition) (Kazmierczak et al. 2020). Thus, our results for a modest effect of AmpDh3

contributing to the competition capacity of PA would be in line with these last results. Meanwhile, however, other works have shown that in other strains of PA such as PA14, the H2-T6SS is the dominant antibacterial weapon (Allsopp et al., 2017). Thus, it would be very interesting to repeat our assays in the future with PA14, probably revealing a more important effect of AmpDh3 for competitiveness. In any case, returning to our PAO1 background, the impact on the competence loss of the double mutant PAΔretSΔADh3 relative to the single mutant PAΔretS was quite modest (CI reductions of ca. 50%), in contrast with other studies in which deleting specific T6SS components (for instance the CplV ATP-ases needed for toxin injection) caused a PA competitiveness reduction of several logarithms (Basler et al., 2013; Allsopp et al., 2017; Wang et al., 2020). Moreover, as explained before, the AmpDh3 role in the competitiveness of PA has been shown to be extraordinarily high when challenging with species not related with CF such as *Y. pseudotuberculosis* or *E. coli*, and thus the deletion of PA *ampDh3*, boosted the competitiveness of these species by up to 2 logarithms (Wang et al., 2020). However, our results suggest that AmpDh3 plays a much milder role to compete with CF-related non-fermenting Gram-negative bacilli than what we hypothesized.

Last but not least, the goal of this master's thesis was to have a better understanding of some of the strategies that PA may use to colonize CF patients with such success. If there was a way to discover a target that is necessary for PA to compete well against less aggressive species (which, regrettably, does not seem to be AmpDh3), it would be eventually possible to "disarm" PA with a drug that neutralized this target and its competitiveness, thus displacing PA by other species, that may be easier to treat with conventional antibiotics. This might represent a cutting-edge tactic, more based on interspecies competition, to get rid of PA from CF patients' lungs and replace it with species that are simpler to manage. Thus, seeking to get closer to this end, although plate competition has been employed as an initial experimental strategy, other methods, such as polymicrobial biofilm models, may be more realistic (Manavathu et al., 2014; Cendra & Torrents, 2021). Therefore, in order to rule out the possibility that AmpDh3 was actually more significant in this sort of models, it will be essential in the future to conduct polymicrobial biofilm research including different strains instead of PAO1 solely, such as the abovementioned PA14.

CONCLUSIONS

1) The variable importance of T6SS over the PA competition capacity appears to be dependent on the competing species: significantly more relevant against SM and

less meaningful against AX and BM. More research will be required to understand the bases supporting the milder impact of T6SS over the PA competitiveness against the two latter species.

2) When *retS* is present (entailing a repressed T6SS), AmpDh3 does not appear to influence PA competitiveness at all.

3) AmpDh3 apparently contributes to PA competitiveness against SM, AX and BM when T6SS is activated, although its impact is much lower compared to other studies where the investigators inactivated different critical T6SS components. Moreover, while AmpDh3 has been demonstrated to be an essential weapon for PA competition against *E. coli* and *Y. pseudotuberculosis* in earlier studies, its importance in the competition against Gram-negative non-fermenters relevant for CF is apparently minor.

4) Future research on the strain PA14 will be required to determine whether AmpDh3 is more important in this strain because, apparently the H2-SST6 (by which AmpDh3 is injected to other bacteria) is the PA14 primary competition mechanism, in contrast with PAO1. Finally, it will be essential in the future to conduct experiments with a polymicrobial biofilm model to complement our plate competition assays, since it is likely a closer scenario to what actually occurs in a CF lung.

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ANNEX

BACTERIAL CULTURE MEDIUMS (all compounds from Oxoid)
- LB (Luria-Bertani):
  - Broth: 1 litter of distilled water, 5 g of sodium chloride (NaCl), 5 g of yeast extract, and 10 g of tryptone.
  - Agar: 15 g of bacteriological agar per litter of LB broth.

- MH (Müller-Hinton):
  - Broth: 21 g of dehydrated medium per litter of distilled water.
  - Agar: 15 g of bacteriological agar per litter of MH broth.

PREPARATION OF TBE 5X buffer
- 27.5 g boric acid (Sigma-Aldrich)
- 54 g TRIS (Sigma-Aldrich)
- 20 ml EDTA (Sigma-Aldrich) pH = 7.6, 0.5 M, making up to 1 L with H2Od.
- Then, the 5x TBE is diluted 10-fold with dH_2O to obtain 0.5x TBE.