Chapter

Pseudomonas aeruginosa Biofilm Lung Infection in Cystic Fibrosis: The Challenge of Persisters

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Abstract

Pseudomonas aeruginosa lung infection is difficult to eradicate due to the multiple (intrinsic and acquired) antibiotic resistance of bacteria and to their ability to produce a thick biofilm. Antibiotic treatment is hampered by poor antibiotic diffusion, efflux pump overexpression and the development of a persistent subpopulation with low metabolic activity. This is a cause for special concern in Cystic Fibrosis (CF) patients, where *P. aeruginosa* lung infection is the chief cause of morbidity and mortality. Combined tobramycin-ciprofloxacin treatment is routinely adopted due to the low frequency of resistant strains and its ostensible ability to control the infection. Nevertheless, symptoms usually recur, mainly due to the antibiotic persisters, which are difficult to detect in routine cultural microbiological assays. This chapter describes the issues involved in the microbiological diagnosis of *P. aeruginosa* lung infection in CF patients and the possible role of subinhibitory antibiotic concentrations in persister development and infection recurrence.

Keywords: *Pseudomonas aeruginosa*, biofilms, antibiotic resistance, bacterial persisters, viable but non-culturable forms, infection recurrence

1. Introduction

Infectious biofilms have long been recognized as a severe clinical problem due to their tolerance to antimicrobials and their successful evasion of host defenses [1]. Their eradication is hampered by a variety of factors that are related to the sessile lifestyle and high cell density typical of biofilms, chiefly the poor diffusion of antibiotics and immune cells, the selection of antibiotic-resistant mutants, the development of intrinsic antibiotic-resistant phenotypes, like small colony variants (SCVs), and the spread of resistance genes among the bacterial populations through horizontal gene transfer (HGT) events. The problem is compounded by the development of antibiotic-unresponsive dormant cells, which upon reaching the late stage of dormancy can become non-culturable [2–4], thus escaping detection by routine culture-based assays [5, 6]. The difficulty of eradicating bacterial biofilms is a key factor in recurrent and chronic infections [1, 7, 8].

The opportunistic pathogen *Pseudomonas aeruginosa* is one of the bacteria most frequently involved in biofilm-related infections. Although most strains are environmental, the pathogen can live in symbiosis with a variety of hosts including plants,

insects and animals. In humans it is an important nosocomial pathogen responsible for a variety of infections that have a strong tendency to recur, particularly in burn patients and in those with lung involvement. Like other opportunistic pathogens, it typically affects immunocompromised individuals [9]. However, the subjects most prone to develop *P. aeruginosa* infection are patients with cystic fibrosis (CF).

2. *P. aeruginosa* biofilms and lung infection in cystic fibrosis

2.1 *P. aeruginosa* biofilms and antibiotic resistance

The ubiquitous presence of *P. aeruginosa*, its prevalence and persistence in clinical settings and its intrinsic resistance to therapeutics are underpinned by an extraordinary arsenal of response mechanisms [10]. In particular, bacteria are protected by biofilms from adverse environmental conditions like phagocytosis, oxidative stress, nutrient/oxygen restriction, metabolic waste accumulation and antimicrobial agents [1, 11]. The matrix – which provides a favorable niche for intense cell–cell interaction and communication and a reservoir of metabolic substances, nutrients and energy [12] – accounts for 90% of the dry weight of the biofilm mass. Its main constituents are extracellular polysaccharides, proteins, extracellular DNA (eDNA), lipids, especially rhamnolipids, and other secreted molecules, such as the siderophores pyoverdine and pyocheclin, pyocyanin and phenazines. The production of all these components is highly regulated by *quorum sensing* (QS). *P. aeruginosa* biofilm development is characterized by the production of large amounts of three types of extracellular polysaccharides: Psl, Pel and alginate. Psl and Pel are the main constituents of the extracellular matrix in non-mucoid strains and are involved in the early stages of biofilm formation and in cell–cell interactions, whereas alginate overproduction is associated with the mucoid phenotype, the hallmark of chronic infection, and is indicative of disease progression and long-term persistence.

Biofilm development is held to be a differentiation process – activated in response to a variety of environmental stimuli – that alters pathogen behavior and results in the adoption of a sessile lifestyle [13]. Biofilms are characterized by an intricate regulation network that induces the development of different bacterial subpopulations and the emergence of antibiotic-resistant variants, which are a typical trait of *P. aeruginosa* biofilms [14]. The heterogeneity of the biofilm bacterial population is associated with the presence of niches with distinctive environmental characteristics that modulate gene expression patterns [15].

Biofilm formation is regulated by a number of redundant mechanisms of which QS is the most widely investigated. Four different QS systems, Las, Rhl, Pqs and Iqs, each characterized by a specific signal molecule and a receptor protein, have been described in *P. aeruginosa*. QS systems are involved in the regulation of several metabolic and pathogenic pathways that have a significant role in bacterial fitness in the environment as well as in the host. Their interplay is governed by a complicated hierarchical network, where the Las system directly regulates the Pqs and the Rhl systems [15].

Additional regulator systems, which sense the changes in the extracellular environment and regulate gene expression accordingly, also seem to be key factors in biofilm population dynamics. The best known is the Gac/Rsm system, which is the main factor controlling the switch from the planktonic to the sessile lifestyle in *P. aeruginosa* [13]. It encompasses two proteins, GacS/GacR, which sense and respond to environmental stimuli, promoting the synthesis of two small RNAs, RsmZ and RsmY, which bind and sequester the post-transcriptional regulator RsmA [16]. It induces the expression of virulence factors and of other genes playing roles

in colonization and acute infection processes, such as the genes involved in motility (synthesis of pili) and in the type III secretion system; at the same time, it represses some genes implicated in chronic infections, such as those encoding the production of alginate and other exopolysaccharides, which constitute the biofilm matrix. RsmA sequestration seems to be a central mechanism in the shift from the planktonic to the biofilm lifestyle [16]. The second messenger c-di-GMP acts through an alternative regulation pathway and seems to promote biofilm development by a variety of routes: repression of motility-related genes, exopolysaccharide overproduction and expression of the adhesin CdrA [17, 18]. RsmA and c-di-GMP share overlapping targets and indirectly regulate each other with antagonistic effects, supporting the notion of a redundant system [19]. The fact that the c-di-GMP positively regulated efflux pump overexpression through *brlR* induction highlights the importance of the messenger in the development of antibiotic resistance/persistence phenotypes [20].

In sessile cells, the action of antibiotics is contrasted by a variety of mechanisms that make them less susceptible to antimicrobials than planktonic cells [21]. Notably, the biofilm matrix acts as a barrier, limiting the diffusion of toxic compounds [22]; in particular, binding to eDNA prevents positively charged antibiotics such as aminoglycosides from penetrating the bacterial cells [23]. Moreover, in biofilm-growing *P. aeruginosa* a wide range of resistance determinants are expressed or upregulated in a biofilm-specific manner [24]. Indeed, overexpression of the efflux pumps – particularly MexAB-OprM and MexXY-OprM – is the main cause of the multiple antibiotic-resistant phenotype [25] that characterizes chronic *P. aeruginosa* infection and contributes to the failure of its eradication in CF patients [26, 27]. The *mexAB-oprM* operon is upregulated in biofilms resistant to azithromycin [28] and fluoroquinolones [29] and also seems to be involved in colistin tolerance, which has been described in actively growing *P. aeruginosa* cells [30]. MexXY-OprM is the main aminoglycoside resistance determinant. It is a typical example of inducible adaptive resistance [31]; this is also demonstrated by the frequent recovery, from chronic patients, of strains bearing mutations in *mexZ*, a repressor gene of the *mex-XY* operon, which is considered as a mutation hotspot in biofilm-growing *P. aeruginosa* and a typical example of convergent evolution of different CF clonal lineages [32, 33]. Other remarkable examples of antibiotic resistance associated with biofilm growth are endogenous AmpC ß-lactamase overexpression and upregulation of the *ndvB* gene [34]; the latter is involved in biofilm-specific synthesis of cyclic glucans, which are responsible for aminoglycoside binding and trapping [35]. Finally, the biofilm is an ideal environment for HGT events [8], which contribute to the spread of resistance determinants. Conjugation events are favored by close contact between cells of different strains and/or species [36]; moreover, it has recently been suggested that *P. aeruginosa* biofilms can achieve a natural competence to acquire both genomic and plasmid DNA [37]. This is a cause of particular concern for chronic CF patients, whose lungs are often colonized by different antibiotic-resistant strains, a condition that has the potential to give rise to multidrug resistance [38].

2.2 *P. aeruginosa* CF lung infection

Cystic Fibrosis is a genetic autosomic disease due to mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which involve a wide range of dysfunctions that alter the airway environment and increase susceptibility to bacterial respiratory infections. *CFTR* gene dysfunction affects epithelial cells, the pancreas (malabsorption), the liver (biliary cirrhosis), the sweat glands (heat shock) and the vas deferens (infertility). Patients with late disease suffer from bronchiectasis, small airway obstruction and progressive respiratory impairment [39]. CF is

characterized by recurrent pulmonary exacerbations. Worsening of the chronic lung infection symptoms (particularly cough and sputum production), increased bacterial load and inflammation and, often, a reduction in FEV1 (forced respiratory volume in 1 second) impair lung function hence quality of life and overall survival.

The identification of effective treatments requires a greater understanding of the factors underpinning the exacerbations. Notably, the lung of CF patients is initially colonized by *Haemophilus influenzae* and *Staphylococcus aureus*; then, patients gradually become susceptible to infection with a variety of environmental Gram-negative bacteria carrying a broad range of constitutive and acquired antibiotic resistance determinants [39]. *P. aeruginosa* is the main pathogen triggering airway inflammation and the leading cause of CF morbidity and mortality [40]. Most CF patients are susceptible to *P. aeruginosa* respiratory infections from infancy. The 30% of them acquire a strain from the environment resulting in acute infections in the first year of life, this rate increases to about 50% before turning 3 years, while mucoid phenotype and chronic infection usually raise from 3 to 16 years [10].

Lung colonization generally involves alternate asymptomatic periods and relapses with progressive tissue deterioration that eventually lead to lung failure and to premature death. Over the years *P. aeruginosa* develops multiple phenotypic variants such as SCVs, mucoid and persistent forms. In particular, SCVs are typically isolated from the lungs of chronic CF patients. They are small (1–3 mm in diameter) usually non-motile and resistant to several classes of antibiotics; produce high amounts of exopolysaccharide and form biofilms that strongly adhere to surfaces [41]. *In vitro* and *in vivo* tests have demonstrated that exposure to sublethal concentrations of antibiotics, such as aminoglycosides, selects for SCVs. In CF patients, prolonged persistent infection, deterioration of pulmonary function and increased antibiotic resistance all correlate with SCVs detection in sputum [41].

P. aeruginosa adaptation to the CF lung environment ultimately results in a mucoid phenotype, a conversion first described by Lam and colleagues [42], which may take several months to years. The mucoid material has subsequently been identified as alginate. In mucoid strains, alginate may favor adhesion to lung epithelial cells, thereby inhibiting clearance. Nutrient restriction, dehydration and suboptimal antibiotic concentrations may result in mucoidity [7, 43]. Host inflammation responses are also believed to contribute to mucoid conversion, a hypothesis that is supported by the absence of mucoid variants among environmental isolates [44].

3. Persister development: antibiotic failure and microbiological diagnosis

3.1 Persistent and viable but non-culturable (VBNC) bacterial forms

P. aeruginosa lung infections tend to be recurrent. Relapses are chiefly due to the development of persisters, bacterial forms that are unsusceptible to antibiotics and often difficult to detect by routine microbiological assays. Persistence has been defined as "the ability of a subset of the bacterial population to survive to a bactericidal antibiotic concentration" [45]. Survival is demonstrated by bacterial growth in culture once the stressor, i.e. antibiotic concentrations several times higher than the minimal inhibitory concentration (MIC), has been removed and nutrients have been restored. Accordingly, the main features distinguishing persisters from resistant cells are the inability of the former cells to grow in presence of antibiotics, though viable and metabolically active, and the lack of heritability [45].

Persisters have been considered as dormant cells that are unaffected by antibiotics [46]. However, lack of significant growth or metabolic activity does not equal

persistence, since the majority (> 99%) of dormant subpopulations are not true persisters. Persistence is a far more complex condition than dormancy [47], it shows an intense metabolic activity despite cell failure to grow or divide. Indeed, starvation-induced persisters produced more ATP per mol of carbon source consumed than nutrient supplied cells did [48]. Accordingly, persister cells seem to be able to catabolize carbon sources, which results in increased electron transport chain activity and membrane potential and increased aminoglycoside uptake [49]. However, although bacterial metabolic processes and persistence are closely related, the mechanisms involved are largely unclear [50].

Antibiotic persistence is not to be confused with antibiotic tolerance. In particular, whereas tolerance involves the whole bacterial population, persistence regards only a subset of specialized cells. Moreover, tolerant cells are killed, even if more slowly than susceptible cells, by high antibiotic doses while persisters are maintained over time (**Figure 1**). Notably, however, the two cell types share the same MIC as susceptible cells [45].

Two types of persisters have been described to date: stochastic and triggered. The former cells constitute a small subpopulation that can be found in all bacterial cultures, even in exponentially growing ones, whereas the latter are induced by environmental as well as host-related stressors. Unfavorable environmental conditions, e.g. nutrient and oxygen depletion, catabolite accumulation and suboptimal pH, which can induce persistence, can occur in the lungs of CF patients, especially in the deepest layers of *P. aeruginosa* biofilms [51]. Repeated antibiotic treatment directed at eradicating chronic infection can contribute to the induction of these specialized bacterial forms [26].

VBNC cells are dormant forms described in several bacterial species, including *P. aeruginosa*. They are characterized by the inability to grow on bacteriological media despite the presence of metabolic activity [52]. VBNC cells share several features with persisters, including a number of inducing factors of which the most common are starvation, oxidative stress, suboptimal salinity and pH and low temperature [52]. Moreover, both phenotypes are highly resilient to antimicrobials. These similarities have led some researchers to conclude that "persister and VBNC cells actually represent subsequent stages of the same cycle of dormancy, adopted by non-sporulating bacteria to survive unfavorable conditions" [52]. According to this theory, stress exposure would induce the development of persisters, which in case of prolonged exposure would turn into VBNC cells, whereas stressor removal

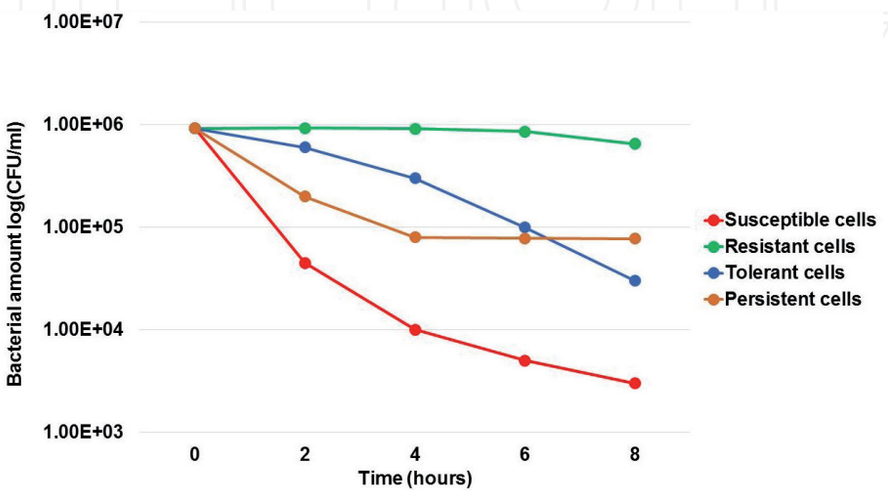

Figure 1.
Behavior of susceptible, resistant, tolerant and persistent bacterial subpopulations treated with antibiotic concentrations exceeding the MIC. CFU: Colony forming unit.

and nutrient restoration would involve recovery of the full metabolic state typical of exponential growth [52, 53]. Unlike culturable persisters, VBNC cells can regain culturability only through the action of specific activators (**Figure 2**), a phenomenon known as resuscitation [54]. The activators can be specific for the bacterial species and even for a single strain; while not completely understood they seem easily found *in vivo* [54].

3.2 VBNC *P. aeruginosa* and issues related to the diagnosis of CF lung infection

In CF patients, the microbiological diagnosis of *P. aeruginosa* lung infection is still performed by culture-based assays, which cannot detect VBNC cells [6]. Such assays involve microorganism isolation using rich (Columbia blood or chocolate) and selective (MacConkey or Pseudomonas) agar followed by isolate identification by biochemical or mass spectrometry analysis [55, 56]. Though effective in diagnosing primary colonization and pulmonary exacerbations, these methods suffer from considerable limitations, first and foremost poor sensitivity, due to the multiple phenotypic variants found in *P. aeruginosa* isolated from chronic CF biofilm-related infections [57].

A variety of stressors, principally nutrient depletion, oxidative and osmotic stress, an acid pH, a strong immune response and the presence of subinhibitory antibiotic concentrations [51, 58], make the CF lung an unfavorable environment for *P. aeruginosa*. The bacterial response involves the development of different phenotypes. The best known is the mucoid phenotype [59], alongside the loss of motility and pigmentation [60], the formation of auxotrophic variants and SCVs [61]. All these phenotypes are characterized by slow growth, which hampers culture-based diagnosis. However, the main problem is detecting VBNC cells. These cells – albeit not necessarily virulent – given suitable conditions can revert to full metabolically active forms capable of quick duplication and full virulence [54], which trigger a new infection. Developing a diagnostic technique that detects these forms is therefore critical to forecast symptom relapse and start early treatment.

3.3 The multifaceted role of antibiotics

Antibiotic treatment can play two different roles as regards persistent cells: it can either select a pre-existing persistent subpopulation or induce the persistent phenotype [45]. The hypothesis has also been advanced that antibiotics exert a biphasic dose-dependent action, i.e. inhibition of bacterial growth at high (≥ the MIC) doses

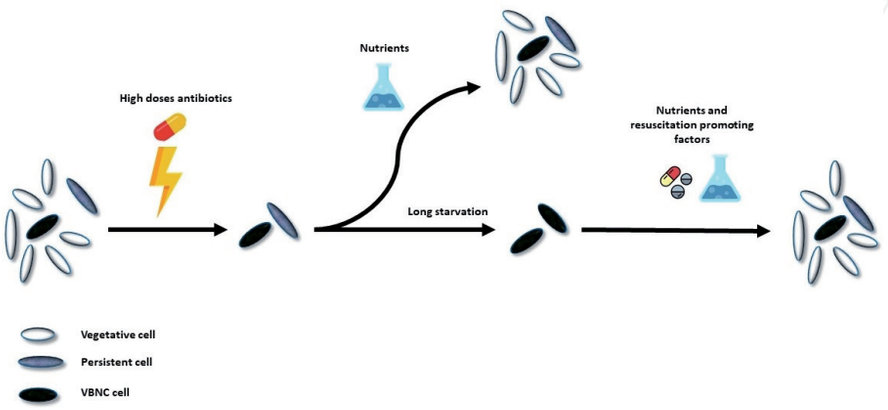

Figure 2.
Differences between culturable persistent and VBNC cells after stressor removal and nutrient restoration. Whereas persistent cells quickly begin to grow and divide again, VBNC cells require exposure to a growth activator (the resuscitation-promoting factor) before regaining full metabolic activity and doubling ability. The progeny of both cell types will contain a mixed population as the starting culture.

and stimulation of a specific bacterial response by acting as a molecular signal at low concentrations (< the MIC), a phenomenon known as hormesis [62].

Failed infection eradication even after prolonged antibiotic treatment is a major clinical problem in patients with microbial biofilms. Antibiotic unresponsiveness has been explained by poor drug penetration in the biofilm matrix and by the development of dormant/persistent cells in the deepest biofilm layers [22]. Since low antibiotic concentrations are probably found for extended periods in the lung of CF patients with intermittent/chronic infection, who undergo repeated antibiotic treatment, the development of persistent forms is likely to be stimulated by the drugs themselves. Evidence to this effect has been reported for different classes of antibiotics, including quinolones and aminoglycosides, although more comprehensive investigations are required to draw firm conclusions.

4. *P. aeruginosa* VBNC cell detection and quantification in CF respiratory samples

4.1 Total live cell detection strategies

Given the wide phenotypic variability of *P. aeruginosa*, encompassing difficult-to grow phenotypes, several culture-independent approaches have been devised to provide reliable infection diagnosis. DNA-based techniques are useful because they are able to detect the whole bacterial population. Most protocols are based on PCR or qPCR assays [63].

To find a suitable target gene on which to base *P. aeruginosa* detection, most protocols have been tested on a variety of bacterial isolates of different origins. The *oprL* gene, encoding a peptidoglycan-associated protein, has long been considered as one of the best targets [64–67]. However, its specificity was questioned when Anuj and colleagues [68] obtained cross-reactions with other species. Notably, the selection of multiple targets is considered as the best approach to *P. aeruginosa* detection, since it excludes false-negative results due to mutations in the amplified gene sequences. The *gyrB* and *ecfX* genes are two other widely used targets. The former gene encodes the DNA gyrase subunit B. Tests against several CF *P. aeruginosa* isolates have identified a species-specific internal sequence [69, 70]. The *ecfX* gene – found in 19 copies/genome – encodes a σ factor belonging to the ECF subfamily, which is involved in the synthesis of proteins with an extracytoplasmic function and seems to play a role in *P. aeruginosa* haem uptake and virulence [71]. The gene has been reported to be specific of *P. aeruginosa* and has been used to achieve its detection in environmental as well as clinical samples [6, 71]. Further proposed targets are the *algD* gene [72] and some 16 s [73] and 23 s rDNA sequences [74].

After reliable detection, a key issue is direct pathogen quantification in sputum samples. Most of the work in this field has been performed after 2010 using specific extraction kits and lysis protocols [66, 67, 74–76].

The main drawback of DNA-based approaches is that they do not detect only live bacterial cells and may be affected by the presence of dead cells as well as by eDNA [77]. An efficient and widely used approach to this problem is to treat samples with propidium monoazide before DNA extraction [66]. The dye penetrates the cells via damaged walls/membranes and binds their DNA; after photo-activation, binding to the nucleic acids prevents DNA polymerase binding, hence DNA amplification in PCR assays. Since live cells commonly have an intact wall, they are not affected by the dye and only their DNA is detected. The same objective can be achieved with other treatments such as ethidium monoazide and DNase [6, 77].

Despite some drawbacks, DNA-based methods provide additional valuable information to the cell culture results when investigating and monitoring *P. aeruginosa* colonization dynamics in the CF lung [78]. Accordingly, extensive metagenomic studies of the CF microbiota have highlighted that persistent cells play a major role in infection chronicization and that persistence is favored by alterations in bacterial gene expression [79], further stressing the value of molecular techniques in routine diagnostics [80, 81].

Another useful technique capable of providing direct bacterial quantification is flow cytometry. Although it has mostly been employed to investigate bacterial physiology and metabolic responses [82], efforts to optimize its quantification ability have made it suitable for some diagnostic applications [83]. In particular, flow cytometry analysis and imaging now enable detection and enumeration of non-culturable and intracellular *P. aeruginosa* cells [84, 85].

Other approaches to detect the whole microbial community of CF lung have also been developed and in the last years indirect detection has been also achieved by metabolomic methods targeting specific bacterial metabolites as pathogen footprints [86].

4.2 Evidence of the presence of VBNC *P. aeruginosa* in CF sputum

The presence of VBNC *P. aeruginosa* cells in the CF lung and in particular their role in infection recurrence are highly controversial. However, the induction of VBNC cells in the CF lung environment currently seems to be the most likely explanation for the failure of infection eradication in the presence of a negative microbiological diagnosis [6].

The first reports of pathogen persistence in patients with negative sputum cultures, published by Schelstraete and Deschaght and colleagues [87], described the swift reappearance of the same *P. aeruginosa* strain, after a brief interval of ostensible resolution, in patients treated with eradication therapy. Deschaght and co-workers [66] subsequently demonstrated that the pathogen could be detected by qPCR much earlier than by culture assays and that qPCR was able to detect a high percentage (62%) of non-culturable *P. aeruginosa* cells in sputum samples from patients who had received the first week of antibiotic treatment. A discrepancy between culture-based and culture-independent methods has also been reported by Le Gall [75] and Héry-Arnaud [76] who advanced the hypothesis of a shift of bacterial cells to a non-culturable state. A positive qPCR assay preceding a positive culture has also been described by McCulloch and colleagues [74] and, more recently, by Boutin and co-workers [88].

Our group has carried out extensive work to identify and quantify VBNC *P. aeruginosa* cells in CF sputum [6]. Combining two previously published *ecfX*-targeting primers we obtained a new amplicon (145 bp) suitable for qPCR. Testing of the new primer pair against a panel of 115 *P. aeruginosa* strains of different origins and other Gram-negative bacterial species failed to elicit a cross-reaction, confirming the species specificity of the selected target. Moreover – even though the use of a single target gene cannot exclude false-negative results due to target mutations [68] – the *ecfX* sequence yielded a positive PCR result in 111/115 (96.6%) of the *P. aeruginosa* strains and the use of a second target gene (*gyrB*) did not lead to an increase of *P. aeruginosa* detection ([89] unpublished data).

Total DNA was extracted from CF sputum samples using the QIAamp DNA kit (Qiagen, Hilden, Germany) and qPCR assays were performed using a SYBR Green reaction format. The sensitivity of the protocol combining DNA extraction and qPCR was determined by testing *P. aeruginosa*-free sputum samples inoculated with

serial dilutions of log phase *P. aeruginosa* cultures. Protocol sensitivity was 70 cells/ml, which is comparable to the sensitivity of TaqMan probe-based qPCR assays [90]. Its limit of detection, determined by amplifying serial dilutions of a purified *ecfX* amplicon, was 5.2 x 10^{-9} ng/reaction, corresponding to about 140 cells/ml in the original samples.

eDNA interference was excluded by treating samples with DNase I (18 U) before DNA extraction. Preliminary assays were performed using *P. aeruginosa*-free sputum samples inoculated with 10% live and 90% dead *P. aeruginosa* cultures. DNase-treated and untreated aliquots were processed using an in-house crude extraction procedure or the QIAamp extraction kit (**Figure 3**).

The qPCR counts of DNase-treated aliquots always matched the live cell quota (10%) of the *P. aeruginosa* inoculum. As regards the untreated aliquots, they corresponded to the whole *P. aeruginosa* load (100%) when qPCR was performed on crude extracts, whereas qPCR performed on DNA extracted with the QIAamp kit yielded counts that were comparable to those obtained after DNase pretreatment. This is likely due to the fact that the eDNA of dead *P. aeruginosa* cells was too damaged to be efficiently bound and retained in the extraction column. It can thus be assumed that DNA extraction with suitable commercial kits – whether alone or combined with DNase treatment – excluded eDNA and provided reliable quantification of live bacterial cells.

We performed the same procedure in 88 CF sputum samples from 55 patients.

The qPCR and culture-based counts were largely comparable (i.e. 78.41% of all samples, 43.18% culture-negative and 35.23% culture-positive). Notably, the absence of samples that were simultaneously culture-positive and qPCR-negative excluded false negatives. The most interesting results were those where the qPCR count exceeded the culture-based count (11.40% of samples) and those where culture-negative samples showed a qPCR-positive result (10.23%). Given eDNA exclusion by DNase treatment and DNA extraction procedure, the discrepancy was held to reflect the presence of VBNC *P. aeruginosa* cells, in line with data reported by Deschaght [66], Le Gall [75], McCulloch [74] and Boutin [88]. Crucially, 1 and 3 months after the PCR-positive results, the cultures turned positive in 2 patients.

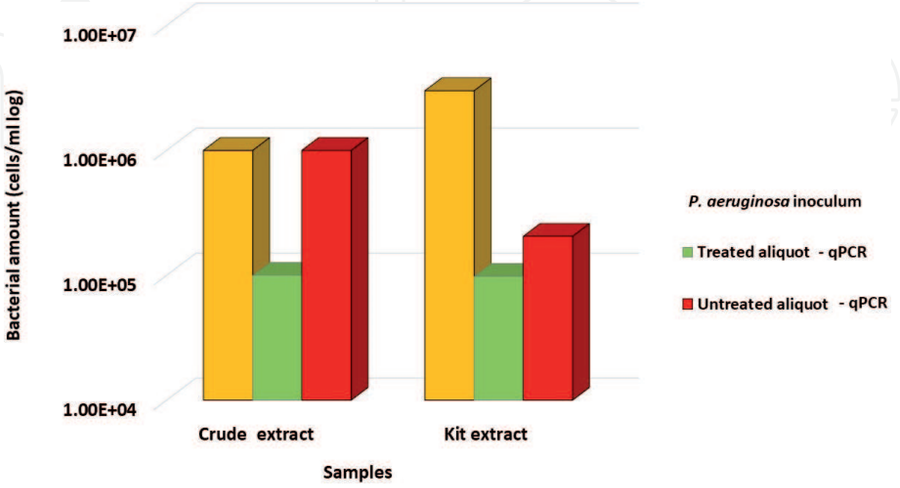

Figure 3.
(Modified from Ref. [6]) Detection of live P. aeruginosa *cells in DNase-treated sputum samples.* P. aeruginosa *abundance was quantified by qPCR in CF sputum samples inoculated with 10% live and 90% dead cultures with/without DNase I pretreatment. DNA was extracted with a crude extraction procedure or with the QIAlamp extraction kit. The qPCR counts were compared to the whole bacterial inoculum.*

5. Evaluation of the possible role of antibiotics and other stressors in triggering VBNC cells and culturable persisters in *P. aeruginosa* biofilms

After demonstrating VBNC *P. aeruginosa* cells in the lungs of CF patients (6), a key issue was to establish the factors that trigger their induction. We therefore examined the role of antibiotic treatments and of some environmental stressors that are found in the CF lung [51, 58], in selecting and maintaining persister cells, particularly VBNC cells.

5.1 Evaluation of the role of sub-MIC of ciprofloxacin and tobramycin in inducing VBNC and culturable persistent cells in starved *P. aeruginosa in vitro* biofilms

The possibility of VBNC cell induction by low antibiotic doses was first explored by our group in starved *S. aureus* biofilms. Greater VBNC cell persistence and survival were found in starved cultures exposed to vancomycin and quinupristin/dalfopristin than in those subjected to starvation alone [91].

Similar experiments were subsequently performed to test VBNC cell induction in *P. aeruginosa* biofilms exposed to starvation, alone or combined with subinhibitory (1/4 x MIC) concentrations of ciprofloxacin or tobramycin [92], which are the most widely used treatments for *P. aeruginosa* lung infection [39, 93]. Biofilms of the laboratory strain *P. aeruginosa* PAO1 and the CF isolate *P. aeruginosa* C24 were developed in rich medium in 35 mm petri dishes for 48 h at 37°C and then subcultured in non-nutrient (NN) broth, alone or supplemented with sub-MIC concentrations of ciprofloxacin or tobramycin for 170 days. The medium was refreshed once a week. Samples were evaluated at 60, 75, 90, 120, 135, 150 and 170 days. The biofilm content in VBNC cells was determined as follows:

- the culturable population was quantified by plate counts performed on cystine lactose electrolyte-deficient (CLED) agar after incubation for 24, 28 and 72 h at 37°C;
- total viable cells (TVCs) were expressed as the average of the counts obtained from *ecfX*-qPCR and flow cytometry assays after live/dead staining using SYBR Green 1x and propidium iodide 40 μg/ml;
- the number of VBNC cells was determined as the difference between TVCs and culturable cells (only differences ≥0.5 log were considered).

The results are reported in **Figure 4** and are expressed as percentage of TVCs.

Unlike the *S. aureus* biofilms, a culturable *P. aeruginosa* subpopulation, which can be defined as triggered persisters, was detected throughout the experiment. A VBNC subpopulation also developed and was more abundant in presence of the antibiotics. In particular, a subset of VBNC *P. aeruginosa* PAO1 cells was detected in all conditions and gradually declined in starved and ciprofloxacin-exposed biofilms; in contrast, the VBNC subpopulation triggered by sub-MIC tobramycin exceeded 90% of TVCs until the end of the experiment. In *P. aeruginosa* C24, starvation alone induced a discontinuous VBNC subpopulation; starvation and ciprofloxacin triggered a persister population only between 75 and 135 days; and starvation and tobramycin induced a constant VBNC population whose abundance was similar to the one determined in the PAO1 strain at the end of the experiment.

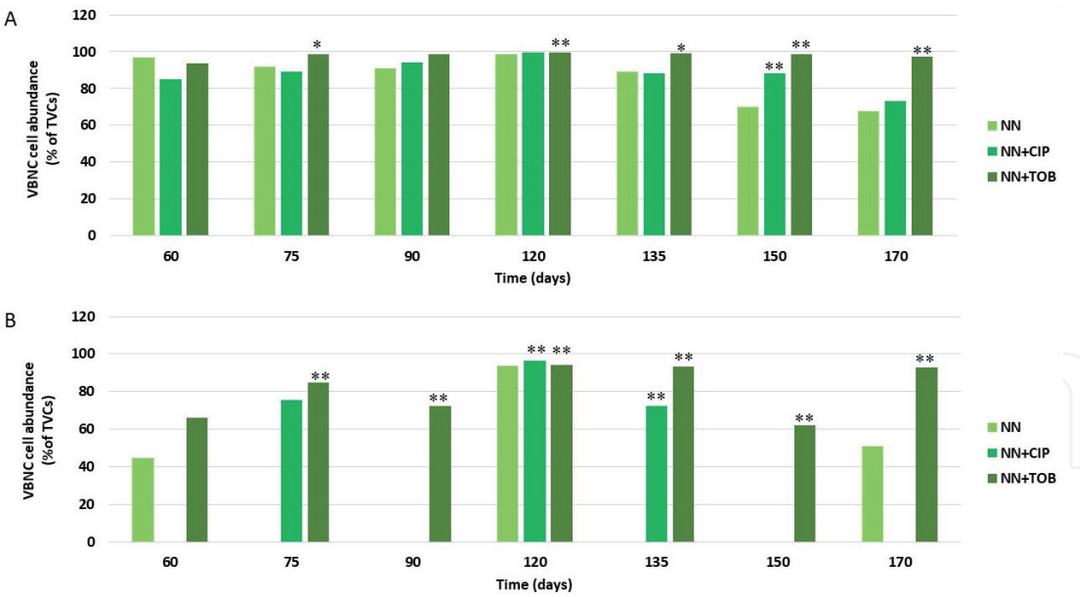

Figure 4.
(Modified from Ref. [92]). VBNC P. aeruginosa *cell abundance in starved biofilms exposed to sub-MIC antibiotic concentrations. The VBNC cell subpopulation was assessed at specific time points in* P. aeruginosa *PAO1 (A) and C24 (B) biofilms exposed to starvation (NN) or starvation+ ¼ x MIC ciprofloxacin (NN + CIP) or tobramycin (NN + TOB). VBNC cells were the difference between total viable cells (TVCs) and plate counts (only differences ≥0.5 log were considered). Cell abundance was compared in the three stress conditions. * = $p < 0.05$, ** = $p < 0.001$.*

These data suggest that sub-MIC concentrations of tobramycin (not ciprofloxacin) play a strong role in inducing VBNC *P. aeruginosa* and are in line with previous reports of the contribution of protein synthesis inhibitors [94] and aminoglycosides [95] to VBNC cell induction. The demonstration of the role of toxin-antitoxin modules [96] and ribosome hibernation [3] in persisters development, exerted via a reduction of protein synthesis, further supports the observed behavior of tobramycin.

After documenting the role of subinhibitory drug concentrations in VBNC cell induction and maintenance, we examined the effectiveness of high antibiotic concentrations on *P. aeruginosa* biofilm eradication by evaluating the abundance of persistent and VBNC cells in mature *P. aeruginosa* PAO1 biofilms exposed to 1000 x MIC/100 x MBEC (minimal biofilm eradication concentration) tobramycin for 24 h (**Figure 5**).

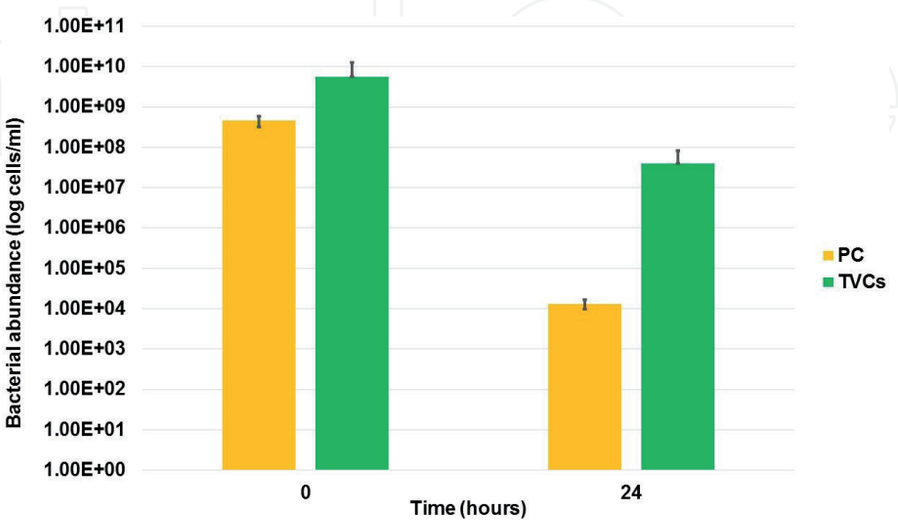

Figure 5.
Biofilm persistence to tobramycin treatment. 24-hour-old P. aeruginosa *PAO1 biofilms were exposed for 24 h to tobramycin 1000 x MIC and assessed for their content in culturable persisters and VBNC cells before and after antibiotic treatment. Persisters were determined by plate count (PC), whereas total viable cells (TVCs) were determined by* ecfX-*qPCR and live/dead flow cytometry. The VBNC population was the difference between TVCs and PCs. The results are given as the average of three biological replicates ± standard deviation.*

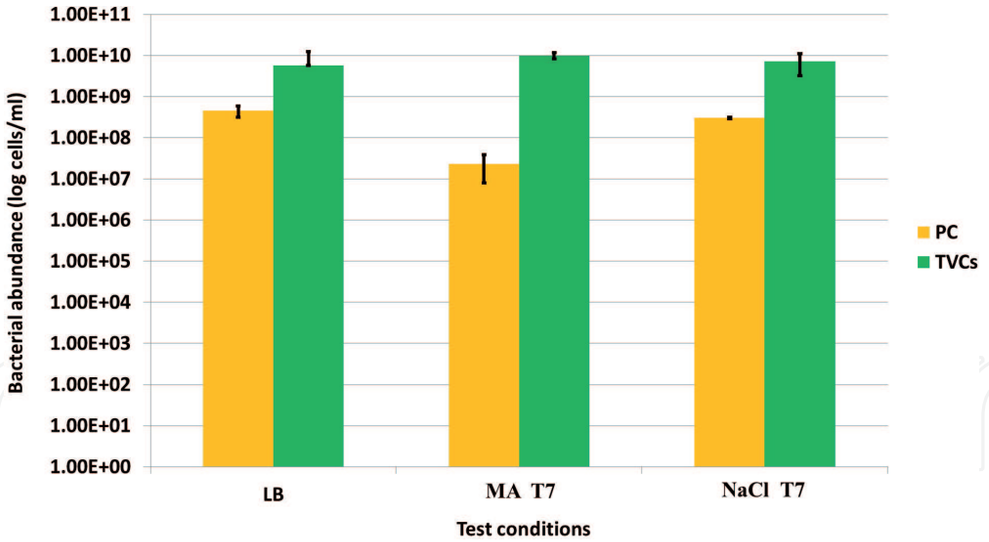

Figure 6.
Induction of VBNC P. aeruginosa *cells in biofilms exposed to environmental stress factors.* P. aeruginosa *PAO1 biofilms were grown in Luria Bertani broth, with metabolite accumulation (MA T7) or in LB + 13 g/l NaCl for 7 days (NaCl T7). Culturable cells were determined by plate count (PC) and total viable cells (TVCs) were determined by qPCR/flow cytometry assays. VBNC cells were the difference between TVCs and culturable cells. These values were compared to those determined in a 24-hour-old biofilm grown in Luria Bertani (LB) broth. The results are average of three biological replicates ± standard deviation.*

As expected, the high tobramycin concentrations chiefly affected the culturable population, which showed a reduction >4 log, whereas the TVC counts showed a 2 log reduction, highlighting the presence of more than 1 x 10^7 VBNC cells/ml after 24-hours exposure to 1000 x MIC tobramycin.

5.2 Evaluation of the possible involvement of additional stressors found in the CF lung

Finally, we examined the possible contribution of further environmental factors – especially the high salinity and metabolite accumulation that are found in the CF lung [51, 58], – in the induction of persistent and VBNC *P. aeruginosa* cells.

Culturable cells and TVCs were counted as described above in *P. aeruginosa* PAO1 biofilms grown for 7 days in Luria Bertani (LB) broth, alone or added with 13 g/l NaCl. The counts were compared to those of a mature 24-hour-old biofilm (**Figure 6**).

As shown in the diagram, *P. aeruginosa* biofilms tolerate and adapt to the high salinity found in the CF lung, since exposure to this stressor for 7 days failed to induce a significant difference in culturable and VBNC cell amount compared to the control condition. In contrast, the plate counts demonstrated a difference of 1 log between 7-day-old and 24-hour-old LB biofilms, whereas the number of TVCs at the two time points was not significantly different. This indicates a shift of *P. aeruginosa* cells to the VBNC state in biofilms grown for 7 days in LB medium, where bacterial metabolites accumulate. Most likely, nutrient reduction and waste accumulation induce a major shift to the persistent state, as also demonstrated for the VBNC cells in biofilms maintained in NN broth.

6. Conclusions

The generation of persistent cell subpopulations is a bacterial survival strategy against adverse environmental conditions [2]. Whereas stochastic persisters are rare, external stressors can convert most bacterial population into persistent cells [45].

In infectious biofilms, a combination of stress factors can induce the development of persistent forms which can trigger infection recurrence. This is a cause of special concern in *P. aeruginosa* CF lung infection, where VBNC cells undermine treatment and hamper microbiological diagnosis, which is still routinely performed by culture-based assays. A routine diagnostic workup including culture-independent approaches should thus be urgently adopted.

The evidence described in this chapter demonstrates that VBNC *P. aeruginosa* cells are found in sputum samples from CF patients and that several months after a culture-negative and qPCR-positive assay some patients experience infection recurrence and have culture-positive sputum samples. These data also highlight the reliability of qPCR in detecting the whole bacterial population, including the phenotypic variants that are missed by culture-based assays. Notably, flow cytometry has demonstrated the accuracy of the *ecfX*-targeting qPCR protocol in detecting all viable *P. aeruginosa* cells and can provide a sound alternative for routine monitoring of the infection. Together, qPCR and flow cytometry supply a clear picture of *P. aeruginosa* population dynamics in the lungs of CF patients with intermittent and chronic infection and can be harnessed to monitor the effectiveness of the antibiotic therapy and to foster the development of new eradication treatments.

The two techniques have enabled us to gain insight into the role of antibiotics in VBNC cell development and infection persistence. Notably, whereas high antibiotic doses can select persistent subpopulations, subinhibitory concentrations – which are found in the CF lung between treatment cycles and in the deepest biofilm layers [51, 58] – can stimulate the development of persistent phenotypic variants, including VBNC cells [53]. Besides the fact that starvation proved to be a necessary condition for VBNC cell induction in our *in vitro* biofilms, our findings highlight a different behavior of tobramycin and ciprofloxacin. Although large amounts of VBNC cells were induced in all test conditions over the first 120 days, their number was maintained more consistently in presence of tobramycin, whereas ciprofloxacin exerted a discontinuous effect similar to the one of starvation alone. These findings can partly be explained by the ability of tobramycin to act as a signal molecule that interferes with QS signals, thus modulating gene expression in biofilm-growing *P. aeruginosa* [58], and by its adverse effect on protein synthesis via ribosome binding [3].

In conclusion, more detailed information on the main gene pathways and persistence regulators and on the effects of different antibiotics is essential to meet the challenge of antibiotic-resilient *P. aeruginosa* infectious biofilms and the eradication of CF lung infection.

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