POLYTECHNIC UNIVERSITY OF MARCHE

DEPARTMENT OF LIFE AND ENVIRONMENTAL SCIENCES

PhD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES
Curriculum Biomolecular Sciences
XXX° CYCLE

Unravelling antibiotic resistance and persistence of *Pseudomonas aeruginosa* cystic fibrosis pulmonary infections

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February 2018

ABSTRACT

Pseudomonas aeruginosa pulmonary infection, often chronic and characterized by recurrent exacerbations, is the main cause of mortality in cystic fibrosis. Although its main dynamics have been defined, many features still represent a riddle for the microbiological research, in particular: the insurgence of possible persister or dormant bacterial forms, possibly involved in the infection recurrence, and the identification of their inducing factors and regulatory genetic pathways; the characterization of the main antibiotic resistance mechanisms and of their putative inhibitors, to develop novel antibiotic treatments against multidrug resistant *P. aeruginosa* strains.

This thesis project has allowed the investigation of these topics, providing the following results: I) the development of an efficient and reliable molecular diagnostic protocol, able to detect *P. aeruginosa* presence, even the viable but non-culturable forms, in cystic fibrosis sputum samples; II) these forms have been subsequently induced in *in vitro P. aeruginosa* biofilms, exposed to subinhibitory concentrations of antibiotics usually adopted in *P. aeruginosa* infections treatment; III) the study of the role of specific regulatory genetic pathways in the formation of bacterial persister cell forms, responsible for the infection recurrence; IV) the definition of the contribution of efflux pumps to *P. aeruginosa* antibiotic resistance and the identification of 3 putative efflux pumps inhibitors, to be used in combination with antibiotics against the main efflux systems of this pathogen in multidrug resistant strains.

The obtained data have provided new insights on the dynamics of *P. aeruginosa* pulmonary infections in cystic fibrosis and allow the development of new therapeutic protocols, to limit the pathogen infectiveness and mortality and to assure a better life expectansy to cystic fibrosis patients.

TABLE OF CONTENTS

INTRODUCTION

1. THE GENUS *Pseudomonas*

The genus *Pseudomonas* includes a wide range of bacterial species, classified as Gammaproteobacteria. They are adapted to live in various environmental niches, including soil (Bergmark et al., 2012), water (Jia et al., 2017), animals (Baldissera et al., 2017; Sperber et al., 2017) and occasionally humans.

The genus was first described by Migula (1894) and over years has raised a progressive interest due to its involvement in several areas of biology, from medicine and health care (Lavenir et al., 2007) to ecology and bioremediation (Ma et al., 2017) and to chemistry (Sanni et al., 2015). The general description of *Pseudomonas* is a Gram-negative, aerobic, non-sporulated rod, motile thanks to polar flagella (Peix et al., 2009); however, over years several informations and new criteria have been provided to better define the genus features: the first consistent study about *Pseudomonas* species was performed by Stanier (Stanier et al., 1966) who indicated their main nutritional, chemical and genotypic features. The DNA composition was further elucidated by Mandel (1966) and the following studies performed by Palleroni (1973), based on RNA-DNA relatedness, finally allowed to divide the genus in five rRNA groups; among these, only the rRNA group I was considered *Pseudomonas*. In the end, the development of the molecular techniques and the sequencing of the 16S ribosomial RNA clearly identified the species belonging to the genus *Pseudomonas sensu strictu* and distinguished very close related genus, like *Stenothrophomonas* (Palleroni and Bradbury, 1993) or *Bulkholderia* (Yabuuchi et al., 1992).

The *Pseudomonas* genus characteristics were systematically described by Krieg and Noel in the first volume of Bergey's Manual of Systematic Bacteriology (1984), where it was reported as composed by Gram-negative rod-shaped bacteria, provided with one or more flagella (responsible for motility), aerobic-microaerophylic, non-spore forming and non-fermenting. They can produce a

wide range of pigmentations, including pyoverdine (Schalk and Guillon, 2013), pyocyanin (Hariri et al., 2017), pyorubin (Lo et al., 2016) and pyomelanin (Hocquet et al., 2016). From a biochemical point of view, *Pseudomonas* bacteria result oxidase positive, citrate positive, β-hemolytic (Okino and Ito, 2016), indole negative, methyl red negative, Voges-Proskauer test negative.

They are characterized by a wide genome, whose major part encodes for regulatory sequences, that allow them to adapt to various environments and to counteract sudden environmental modifactions. *P. aeruginosa*, the most known *Pseudomonas* species, presents a 6,624,403 bp genome, with a 66.6% G+C content and around 468 genes coding for transcription regulators (Stover et al., 2000), the highest proportion of regulatory genes observed in a sequenced bacterial chromosome. The presence of other extrachromosomic DNA molecules, such as plasmids (Michalska et al., 2014), or genetic mobile elements (Yamane et al., 2004), contributes to enhance *Pseudomonas* ability to survive in a wide range of ecological niches, as well as to colonize several hosts.

Excluding *P. aeruginosa*, only a few species have been characterized as they do not result significant in the clinical environment, but they are mainly involved in plant pathologies (Menz et al., 2017) or used in plant-growth applications (Maroniche et al., 2016).

Nowadays, the *Pseudomonas* species characterization is based on different criteria, both phenotypic and genotypic (Peix et al., 2009). Among the formers:

- Cell shape
- Flagella type
- Different carbon sources use
- Growth in different culture conditions
- Antibiotic substances production
- Exocellular enzymes production
- Antibiotic resistance

- Quinone systems
- Fatty acid profile
- Protein profile
- Polar lipid or polyamine profile
- Siderotype
- Fluorescent spettroscopy type

For the genotypic methods the most common are:

- Low molecular weight RNA (5S rRNA or tRNA) characterization
- 16S rRNA sequencing
- Specific housekeeping genes sequencing
- 16S-23S rRNA intergenic spacer sequencing

All these approaches allow an accurate and specific identification, distinguishing even between closely related species, like *P. chlororaphis*, *P. lundensis* and *P. fragi*, and grouping species with determined features in well-defined complexes.

However, *P. aeruginosa* remains the main representative of the entire genus and the most known to man, due to its spread in almost all environmental niches, its involvement in veterinary patologies, and, most of all, to its opportunistic relation with the human organism. Among years, the knowledge and the interest about this microorganism, its lifestyle and its role in human pathologies have raised, making it one of the most studied bacteria, and unravelling the main charcteristics regarding its metabolism and pathogenicity.

2. *Pseudomonas aeruginosa*

2.1 Cell morphology and features

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium, 1-5 μm long and 0.5-1.0 μm wide. It presents a high selective outer membrane, that is characterized by a 12-100 times lower permeability compared to *Escherichia coli* (Breidenstein et al, 2011), with few porin proteins; in particular, OprF, the largest porin channel, is present in a limited number, whereas the other representatives, like OprD and OprB, are characterized by smaller channels, preventing the diffusion of toxic compounds. When exposed to toxic substances, including antibiotics, the porin expression can be down-regulated, limiting even more the membrane permeability (Raavi et al., 2017). The major outer-membrane component, the LipoPoliSaccharide (LPS), reports some unique features (Pier, 2007). The Lipid A presents a basic organization, but in laboratory-adapted strains a coexistence of penta-acylated and hexa-acylated (3:1) residues, as well as the isolation of epta-acylated variants, has been reported; moreover, in Cystic Fibrosis (CF) chronic phase isolates, the hexa-acylated forms are the predominant ones. Bound to the lipid A, there is a relatively conserved inner core structure, characterized by tri-phosphate substituents, a feature that has been reported only in this species. Phosphorilation appears to be essential to the bacterium viability, as demonstrated by lethal phenotypes due to mutations to phosphorilation effector proteins (Walsh et al., 2000). Finally, the external core of *P. aeruginosa* LPS is present in two isoforms, glycoform 1 and 2, produced by the same strain and differing in the position of specialised residues, like 3D-glucose, L-rhamnose and N-alanylated-galactosamine (Sadovskaya et al., 2000). Glycoform 2, in particular, is the structure that binds the O antigen, used for serotyping. *P. aeruginosa* strains can be divided, according to the International Antigenic Scheme (IATS) in 20 serotypes, among whom IATS-O1 (serogroup 2), IATS-

O6 and IATS-O11 are considered responsible for 70% of infections (Lu et al., 2014). Indeed, a relation virulence-serotype was described by Faure et al. (2003), who underlined how the expression of specific extracellular toxic compounds was correlated with the IATS-O11 serotype. Some clinical CF *P. aeruginosa* strains, known as LPS-rough strains, have been reported to accumulate mutations in LPS biosyntethic genes and result often defective for the O antigen (Evans et al., 1994). LPS is deeply involved in various *P. aeruginosa* features, mostly contributing to its virulence as a key factor during infection development:

- It is important in cell-surface adhesion and thus it contributes to biofilm formation in its initial steps (Ruhal et al., 2015);
- It represents the target for phage adhesion and mutation in the lipid A contributes to phage-resistant phenotype (Latino et al., 2017);
- It mediates host immune system evasion (McCarthy et al., 2017) and induces inflammatory state, acting as endotoxin (Liu et al., 2017).

Other major protein complexes present in the outer membrane are constituted by the secretion systems and the efflux pumps. The secretion systems are maybe the most powerful weapon described in *P. aeruginosa*, as able to inject direclty into the host cells toxic peptides (Chakravarty et al., 2017); they are particularly influent in acute infections and are positevely controlled by different transcriptional and post-transciptional regulators, like the Gac/Rsm system (Moscoso et al., 2012).

Two main secretion system have been characterized in *P. aeruginosa*. The Type Three Secretion System (T3SS) is the main virulence determinant and is often used as a biomarker for acute infections detection (Juan et al., 2017). It presents a common and conserved structure among Gram-negative bacteria, as shown in Fig. 1, derived from the flagellum and constituted by a combined secretion and translocation apparartus (Anantharajah et al., 2016).

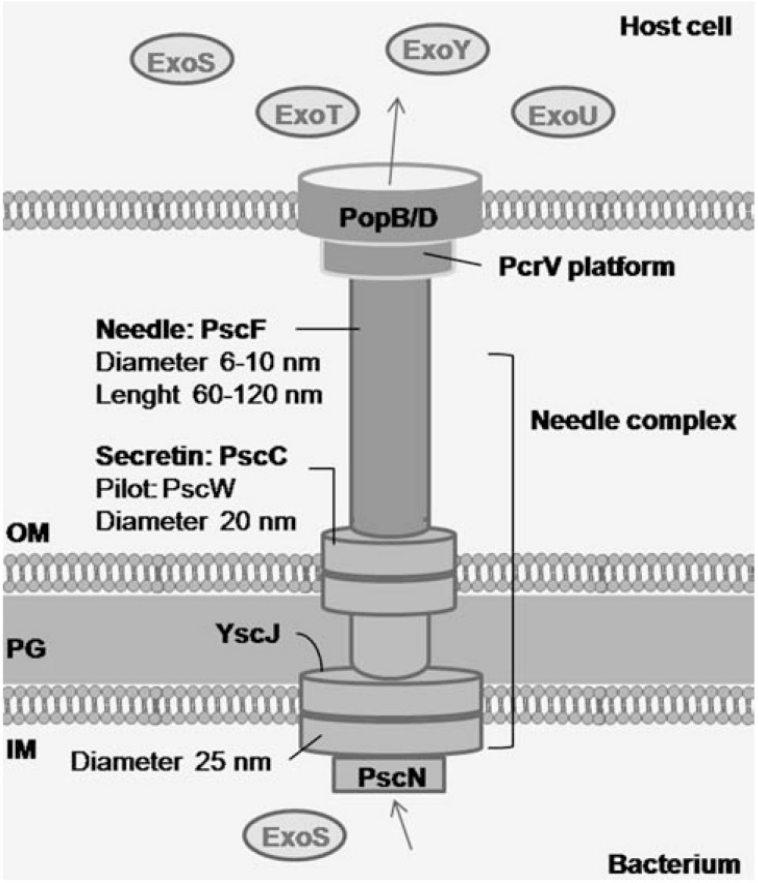

Fig. 1. Schematic structure of the *P. aeruginosa* T3SS, showing the needle complex and the translocation apparatus.

The former, consisting of around 25 different proteins and even known as needle complex, is divided in a needle and a multi-ring basal body, spanning in all the cell layers and anchoring the needle itself to the outer membrane. The translocation apparatus is composed by the two proteins PopB and PopD, that form a pore into the host cell membrane for the subsequent injection, and the PcrV protein, that allows the functional assembly of the complex. The T3SS is responsible for the direct injecton of different exoenzymes into the cell cytoplasm, determining the cytotoxic effect; this action is particurarly effective on the neutrophiles, where it induces apoptotis, allowing the survival of the bacterial cells (Sun et al., 2012). In CF, *P. aeruginosa* strains over-expressing the T3SS are recovered especially in initial stages of infections, and in animal

pneumonia models the presence of this virulence determinant is often associated with an higher rate of mortality, compared to other strains lacking or defective of its action (Galle et al., 2012). In the latest years, various attempts to identify T3SS effectors inhibitors or antibodies have been performed, and currently a small molecule and a bifunctional antibody are in phase II clinical trial as possible T3SS inhibitors in anti-*Pseudomonas* therapies (Anantharajah et al., 2016).

Type 6 secretion system (T6SS) is involved in *P. aeruginosa* warfare for competition with other bacteria in the colonization of environmental and human body niches (Allsopp et al., 2017); this microorganism encodes for three clusters of T6SS, specifically H1-/H2-/H3-T6SS. Among them, while H1-T6SS has a specific anti-prokaryotic action, H2- and H3-T6SS have been demonstrated to target even eukaryotic cells (Sana et al., 2016). Typical T6SS toxins comprehend lipase factors and other lytic enzymes for the bacterial membranes. This system has been reported as controlled by RsmA, belonging to the Gac/Rsm system, and expressed in specific situations, regarding biofilm production and development in chronic infections (Li et al., 2013). Moreover, one work reported how extracellular DNA (eDNA), a key structural factor of *P. aeruginosa* biofilms, can activate T6SS, favoring the pathogen in prevaling during polimicrobial infections (Wilton et al., 2016); this behaviour can give new insights regarding the different bacteria dynamics in CF lung infections, where after a certain age, *P. aeruginosa* becomes the main colonizers of respiratory airways.

Efflux pumps are well-characterized protein complexes in the Gram-negative outer membrane. First dedicated to expell catabolites or toxic compounds from the bacterial cell, they have evolved, under the constant pressure of antibiotic therapy, to extrude even these molecules directly out of cells (Webber and Piddock, 2003).

The efflux pumps are constitutively present in *P. aeruginosa* core genome, so they represent one of the main mechanisms of the intrinsic antibiotic resistance. Their role, as well as their efficacy, is dependent on their expression: some of them result active only when induced and in normal conditions they can only partially counteract the antibiotic action. However, a specific and peculiar tract of the chronic infections is the accumulation of mutations and it has been reported that the regulators of these genes are among the hotspots for positive mutations (Ramanathan et al., 2017).

Their intrinsic and ancestral role is partially conserved, since some of them, especially the pump MexAB-OprM, have been reported to contribue to biofilm formation (Piddock, 2006), by extruding in the culture media signal molecules, able to induce an active response in the growing bacterial population.

In the recent years, a growing interest has raised in determining new molecules or compounds acting like Efflux Pumps Inhibitors (EPIs) (Rampioni et al., 2017), both of natural and semi-synthetic origin, to be used in association therapies with antibiotics, in order to counteract this multi-resistance mechanism; some leading compounds have been identified, but most of them present a too high toxicity to be used *in vivo*.

P. aeruginosa typically presents two kinds of adherence factors, 1-3 unipolar flagella and type IV pili. Flagella are mainly responsible for this pathogen motility and allow it to exhibit different kinds of movements and to colonize different surfaces, both biotic and abiotic. It has, indeed, been reported that *P. aeruginosa* adhesion to hydrophobic surfaces is mediated by flagella more than by pili (Bruzaud et al., 2015), as well as its ability to translocate across the intestinal epithelial cell barrier (Okuda et al., 2017); they are included among the *Pseudomonas* virulence factors and result very important in chemotaxis (Schwarzer et al., 2016). Their expression is regulated by different genes, in particular the ones involved in c-di-GMP levels control (Valentini et al., 2016;

Rossello et al., 2017). In the latest years, flagella have become of great interest considering anti-*Pseudomonas* treatments, since it has been demonstrated that only flagellated and motile bacteria can induce the formation of Neutrophiles Extracellular Traps (Floyd et al., 2016) and that both flagellin and pilin proteins can promote immunization against Multi-Drug Resistant (MDR) *P. aeruginosa* (Korpi et al., 2016).

Type IV pili are long, thin and flexible proteins and constitute the primary virulence factors in *P. aeruginosa*, allowing the adhesion to hydrophilic surfaces, especially the epithelial tissues (Faezi, 2017). Their role is guaranted by a disulfide loop domain in the pilin protein (Farinha et al., 1994), whose mutation causes the loss of the ability of infecting human cells and reduces bacterial viability. Moreover, they are essential in the first stages of biofilm formation, mediating both adhesion to surfaces and allowing swarming and swimming motilities, pivotal to develop a mature biofilm (Otton et al., 2017). The pili production and action are controlled by several genes, in particular those belonging to the *pil* (Jain et al., 2017) and *fim* clusters (Wehbi et al., 2011), and are influenced by the second messenger c-di-GMP levels and exopolysaccharide production (Ribbe et al., 2017). Interestingly, although both types of appendices are involved in adhesion to human epithelial tissues, pili and flagella adopt different mechanisms to colonize cell layers, in particular flagella recognising heparan sulfate proteoglycans, typical of the basolateral side of human epithelial cells, and pili binding N-glycans in the apical side (Bucior et al., 2012). This appears like a convenient strategy, allowing the pathogen to colonize human cells by exploiting different signals and receptors and avoiding negative effects caused by single-target mutations.

Regarding *P. aeruginosa* motility, this bacterium exhibits 3 kinds of movements (O'May and Tufenkji 2011), all involved in its pathogenicity and in the bacterial response to environmental stimuli:

- Swarming: surface associated motility, based on hyperflagellation or multiple pili, it inolves a massive population movement, the action of biosurfactans (i.e. rhamnolipides) and allows the fast colonization of a surface (Yang et al., 2017) through a specific dendritic pattern, in contrast to other bacteria species that use a monolayer scheme;
- Swimming: flagellum-mediated movement in liquid or low-viscosity conditions, it does not require messengers or biosurfactans (Ha et al., 2014). It is deeply involved in colonization, biofilm formation and in chemotaxis and around five different swimming patterns have been described (Vater et al., 2014);
- Twiching: type IV pili dependent motility, controlled by c-AMP levels (Buensuceso et al., 2017), it allows the migration across a surface, forming cells aggregates and contributing to biofilm development (Wozniak and Keyser, 2004). It has been reported even as implicated in oxidative stress response in *P. aeruginosa* (Fonseca et al., 2004).

2.2 Biochemistry

P. aeruginosa is a typical non-fermenting bacterium, a well-know feature that helps its identification in routine culture media, such as McConkey (MC) agar or Cystine, lactose, electrolyte deficient (CLED) agar.

From a biochemical point of view, the pathogen identification is usually obtained through the API 20NE system by BioMérieux (Marcy-l'Étoile, France), reporting these results:

- Nitrate reduction positive
- Indole production negative
- Glucose fermentation negative
- Arginine dehydrolase positive
- Urease negative
- Esculin hydrolysis negative
- Gelatin hydrolisis positive
- B-galactosidase negative
- Glucose assimilation positive
- Arabinose assimilation negative
- Mannose assimilation negative
- Mannitol assimilation positive
- N-acetyl-glucosamine assimilation positive
- Maltose assimilation negative
- Potassium gluconate assimilation positive
- Capric acid assimilation positive
- Adipic acid assimilation positive
- Malic acid assimilation positive
- Citrate assimilation positive
- Phenylacetic acid assimilation negative

- Oxidase positive

It is indeed considered one of the reference organisms in the oxidase test, which is often use for a fast putative identification.

Regarding its nutritional needs, *P. aeruginosa* does not result an esigent or fastidious microorganism: it can grow both in presence and absence of oxygen and it does not require specific factors to develop. Thus, it can be easily cultured in the majority of laboratory culture media and it tends to colonize almost every environmental niche, prevaling over other microorganisms. This versatility is mainly due to a well defined core metabolism. Energy generation is based on oxidative subtrate-catabolism and favourite substrates are short-chain fatty acids, amino acids and polyamines, wherease carbohydrates as glucose are degraded through secondary metabolic ways.

Anyway, *P. aeruginosa* exhibits a great metabolic adaptation, consuming the preferred substrates during the exponential growth phase, whereas sugars are used especially in the stationary phase; moreover, this behaviour is constintent with the production of exopolysaccharides and the biofilm production typical of this growth phase (Frimmersdorf et al., 2010).

A great role in *P. aeruginosa* environment adaptation is played by pigments, that display further effects as virulence factors, mainly involved in oxidative damage of the host tissues (Kujawa et al., 2017). Pyoverdine, in particular, the main pigment conferring *P. aeruginosa* the typical green color, acts mainly as a siderophore, sequestering environmental iron, that constitutes one of the main factors in *Pseudomonas* infection development, as well as in biofilm formation (Visca et al., 2007); the same action causes the *Pseudomonas* prevaling over other microbial species (Sass et al., 2017), since iron quantities are depleted from envinronment and affect microorganisms growth.

At least 60 different isoforms of pyoverdines have been characterized in fluorescent pseudomonads, and their synthesis is a complex pathway, starting in the bacterial cytoplasm and ending by its extrusion in the external environment through a specialized efflux pump, PvdRT-OmpQ (Schalk and Guillon, 2013). Once sinthetised, pyoverdine is traslocated through the outer membrane by the FpvA transporter and is then able to bind the Fe(III) available in the environment; FpvA acts even as receptor for the ferripyoverdine, trasmitting a signal to the bacterial cytoplasm by the FpvR protein and resulting in specific transcription factors activation, responsible not only for an increased iron uptake, but even for the expression of different virulence factors (Minandri et al., 2016).

The pyoverdine system is not the only iron uptake strategy performed by *P. aeruginosa*; other well described mechanisms are the Phu and the Has systems (Ochsner et al., 2000) and the TonB-ExbB-ExbD protein complex (Noinaj et al., 2010), indicating the *P. aeruginosa* tend to accumulate iron as essential component of its metabolism. Recently, a new siderophore system, specific for the airway mucus, has been characterized in this microorganism (Gi et al., 2015).

2.3 Pathogenicity

P. aeruginosa is a typical nosocomial opportunistic pathogen, able to cause infections in immunocompromised hosts (de Bentzmann and Plésiat, 2011). It is involved in severe infections in several districts of human body, like respiratory airways, urinary tract (Zhang et al., 2014), eyes (Yamaguchi et al., 2014), skin, circulatory system (Peña et al., 2015) and auditory tract, taking adventage of immunity deficiency, as for example in CF, or specific host conditions, like injured, burned or traumatized subjects (Neyestanaki et al., 2014). These features, united with an impressive ability to develop antibiotic resistance, make it a health care problem, since it can be acquired both from the environment and from hospitals. *P. aeruginosa* represents, indeed, one of the most feared pathogens in intensive care units and it is often isolated from hospitalized patients (Venier et al., 2014).

However, CF *P. aeruginosa* strains do not belong to a unique, well defined clone, a fact that makes clinician suppose that the infection is most aquired in the community and results policlonal.

P. aeruginosa pathogenicity is due to a wide range of virulence factors and strategies.

As already cited, type IV pili and flagella play an important role for initial adhesion to a surface and biofilm formation. It has been demonstrated that *P. aeruginosa* tropism is directed to polarized cell monolayers, like in epithelial tissues, in specific spots

characterized by multicellular junctions (Capasso et al., 2016). In particular, it interacts specifically with the apical side of cell layers, the other sides showing resistance to *P. aeruginosa* attachment, forming aggregates that are often internalized using a specific mechanism (Lepanto et al., 2011). A good adhesion, both to a surface and to other *P. aeruginosa* cells, is then pivotal to

favor the communication and interaction mechanisms which ultimately result in the biofilm development.

In infecting a host, this bacterium has developed several strategies to evade the immune system action. Excluding biofilm, that represents per sè an efficient barrier both to immune cells action and to subsequent antiobiotic treatments, *P. aeruginosa* presents various evasion mechanisms:

- Production of Pls and other polysaccharides, compromising neutrophiles phagocytosis (Thanabalasuriar et al., 2017)
- Activation of inflammation mechanisms typical for intracellular pathogens through T3SS (Faure et al., 2014) or inducing macrophages autophagy (Deng et al., 2015)
- LPS modification, in particular Lipid A modification and presence of few, short or absent O antygens, especially during chronic infections (Maldonado et al., 2016)
- Macrophages and polymorphonuclear leukocytes lysis through rhamnolipids, a strategy occuring mainly during biofilm growth (Alhede et al., 2014)
- Complement inactivation through the alkaline protease (Laarman et al., 2012)
- T cell suppression by induction of myeloid-derived suppressor cells (Rieber et al., 2013)

P. aeruginosa pigments act as virulence factors as well, being most involved in oxidative damage. Pyocyanin in particular is considered a marker of severe infections, being able to inhibit mammalian cell respirations and the respiratory-tract cilia movement (Nowroozi et al., 2012). It has been reported even to show antimicrobial activity and contributes to eliminate bacterial competition. Pyorubin is implicated in oxidative stress as well and in the latests years a new red pigment, aeruginosin A, has been described as a new member of the

phenazine group of pigments (Abu et al., 2013). Both pigments are controlled by c-di-GMP and indirectly by the *fliA* gene (Lo et al., 2016), modulating the second messenger levels. Pyomelanin is mainly characteristic of chronic CF *P. aeruginosa* isoltes; interestingly, Hocquet et al. reported (2016) that pyomelanin producing strains are overall mutants, featured by big deletions in their genome, affecting both their growth fitness and their metabolic spectrum. However, these strains are characterized by a high resistance to intraspecific pyocins and showed to inhibit other *P. aeruginosa* strains, suggesting a mutation positively selected during infections development to compete with other bacterial strains.

The role of pyoverdine as siderophore is well known and characterized, as well as its importance in guaranting *P. aeruginosa* fitness during infections. Several pathways have been described to regulate its production: the main control is executed by the Ferric Uptake Regulator (FUR) which can regulate siderophores genes directly or indirectly, through the action of specific σ-transcription factors. In the case of pyoverdine, the *fur* gene acts regulating the *pvdS* gene, the σ-factor involved in the pyoverdine (*pvd*) gene cluster transcription. It has been reported that mutations in the DNA binding motif of Fur can enhance pyoverdine production (Valesová et al., 2013).

Another possible regulation mechanism has been proposed, that results Fur-independent and is based on the Gac/Rsm system (Frangipiani et al., 2014); according to this model, the Gac/Rsm system acts by modulation of the c-di-GMP levels, that are supposed to regulate the *pvd* gene cluster to cope with environmental iron depletion.

Regarding other virulence factors, *P. aeruginosa* is famous for the production of a wide range of exoenzymes and toxins; 7 secreted proteases have been described (Kessler and Safrin, 2014):

- Elastase B, enconded by the *lasB* gene, allows the epithelial cell penetration (Cowell et al., 2003) and causes cell damage by degrading

collagen, fibrinogen, elastin and opsonin receptors (van't Wout et al., 2015)

- Elastase A, encoded by the *lasA* gene, showing staphylolytic activity, is an endopeptidase; it affects the epithelial cell heparan sulfate proteoglycan and it is involved in elastin degradation in the connetive tissue (Spencer et al., 2010). Moreover, it has been reported as one of the main effectors produced by high virulent *P. aeruginosa* strains isolated in chronic CF infections, coexistent of less virulent strains (O'Brien et al., 2017)
- Alkalyne protease, encoded by the gene *aprA*, is involved in cell damage and immune sysem evasion, by the induction of endoplasmic reticulum stress (van't Wout et al., 2015) and the degradation of flagellin to prevent the immune system recognition (Casilag et al., 2015). It has been reported to contribute even to pyocyanin production (Iiyama et al., 2017)
- Protease IV, encoded by the *piv* gene, is a lysin-specific peptidase and represents a key factor in *P. aeruginosa* virulence. Interestingly, it has been demonstrated to be activated only after production and secretion by cleavage by other virulence factors, such as LasB elastase, in a finely controlled pattern (Oh et al., 2017)
- The *P. aeruginosa* Small Protease (PASP) is mainly involved in ocular infections, causing corneal erosion by cleaving collagen (Tang et al., 2009); it has been reported to degrade complement C3 component, fibrinogen, antimicrobial peptide LL-37 and constituents of the tear film (Tang et al., 2013)
- The large exoprotease, coded by the *lepA* gene, modulates inflammatory and immune responsed, by cleaving the tethered ligand region of human protease-activated receptors (Kida et al., 2008); it has been reported to cooperate with the hemolytic phospholipase C to influence *P. aeruginosa* growth and infection development (Kida et al., 2011)

- Several aminopeptidases have been described, some of them belonging to the metalloprotease family (Cahan et al., 2001), and involved into the microorganism response to antimicrobial substances, even in the environmental strains (Wu et al., 2014; Tang et al., 2017)

In addiction, *P. aeruginosa* is provided with other two important virulence factors. Exotoxin A constitutes maybe the most known secreted factors, showing ADP-rybosylation activity and affecting the host cell protein synthesis (Amini et al., 2017). It is synthesized as a pre-toxin and, once internalized by the host cell by endocytosis, is converted into the active toxin by the Furin transmembrane serine protease and causes cell damage. However, it has been demonstrated that inhibiton of Furin is not sufficient to prevent exotoxin A action (Ferguson et al., 2016) and, indeed, a shorter, intracellular, endosome-mediated route has been described (Morlon-Guyot et al., 2009). Its toxicity has been reported to be important both in the airway epithelium (Ferguson et al., 2016), in the liver (Chiu et al., 2009) and in the traslocation process through the intestinal monolayer (Okuda et al., 2017).

The hemolytic phospholipase C has been demonstrated as particularly important in CF pulmonary infections (Wargo et al., 2011). This enzyme, targeting especially phosphatidylcholine and sphingomyeline, is the main responsible for the pulmonary surfactants degradation, affecting lung functionaliy and favoring the development of microbial infections. Moreover, it has been demonstrated to suppress neutrophiles respiratory burst activity, contributing to *P. aeruginosa* persistence in the lung environment (Terada et al., 1999).

The T3SS and T6SS play an important role in the transport of these factors and other effectors directly into the cytoplasm of host cells. In *P. aeruginosa* 4 T3SS effectors have been identified, as deeply involved in the microorganism pathogenicity, ExoS, ExoT, ExoU and ExoY (Lee et al., 2005). ExoS and ExoT are bifunctional proteins, characterized by a N-terminal G-activating protein

domain and a C-terminal ADP-ribosylating domain, and are involved in cytoskeleton and signal pathway damages in the host cell. ExoY is an adenylate cyclase, whereas ExoU a phospholipase. ExoT and ExoY are generally present in the majority of CF *P. aeruginosa* strains, whereas ExoS and ExoU seem to be mutually exclusive; their distribution among *P. aeruginosa* strains is not different between clinical and environmental isolates, with a trand, especially evidenced in CF isolates, to maintein the *exoS* gene instead of the *exoU* one (Feltman et al., 2001). Consistently with these data, Wareham and Curtis (2007) evidenced that ExoU secreting strains are more common in clinical blood samples than in CF infections, indicating a specific role of the effector in acute infection and in bacteremia development.

Finally, *P. aeruginosa* shows the production of at least 3 types of pyocins (R-,F- and S-type), presenting different structures and activities (Michel-Briand and Baysse, 2002). They are present in the genome of more of 90% of *P. aeruginosa* strains and their expression seems to be upregulated in microaerophilic and anaerobic conditions. This has led to the hypothesis that their role is to allow a specific evolution in mixed cultures and populations, favoring the survailance of specific phenotypes, resistant to the pyocins themselves (Waite and Curtis, 2009).

The presence of all these virulence factors have been correlated with the severity of infections and, especially in the T3SS effectors case, with a poor clinical outcome (Roy-Burman et al., 2001). What is surprising is the positive correlation between the presence of specific virulence factors and antibiotic resistance in chronic infections (Park and al., 2017), even in pneumonia cases (Sawa et al., 2014).

Indeed, two studies have underlined how *P. aeruginosa* cells exposed to antibiotics (Takahashi et al., 2016), especially in biofilms (Oldak and Trafny, 2005), are not inhibited as regards the secretion of virulence factors. This

evidence, probably due to the presence of specific plasmids and other extrachromosomal DNA molecules, is of great importance, since, while allowing to understand the basic mechanisms of *P. aeruginosa* pathology, gives new insights on the microbial persistence after antibiotic treatments.

2.4 Biofilm formation

Bacterial biofilms are mono- or pluri-microbial communities embedded in an exopolysaccharide matrix, secreted by the microbial cells themselves. They are of great importance both in medical and environmental fields, since they represent a winning strategy adopted by bacteria regarding growth and development, the production of specific compounds, the differentiation of specialized bacterial populations and the defense against antimicrobial compounds or the immune system (Høiby et al., 2010 A).

P. aeruginosa is considered the model microorganism for the study of bacterial biofilms, since the biofilm lifestyle is deeply involved in every feature of this pathogen life.

Its architecture is often described as stratified and composed by “towers” or “mushrooms”, referring to specific formations of the matrix. The structure comprehends even small water courses, responsible for oxygen and nutrient delivering to the bacterial cells. Given the presence of multiple layers, these two factors are often present in a gradient, being more concentrated in the external zone and creating, in the internal ones, deep anoxic and nutrient-depleted microenvinronments, where specialized bacterial populations can develop (Sanchez et al. 2013).

The biofilm formation is a complex, multiphasic and fine-regulated process, usually divided in three main stages (Tolker-Nielsen , 2015), represented in Fig.2.

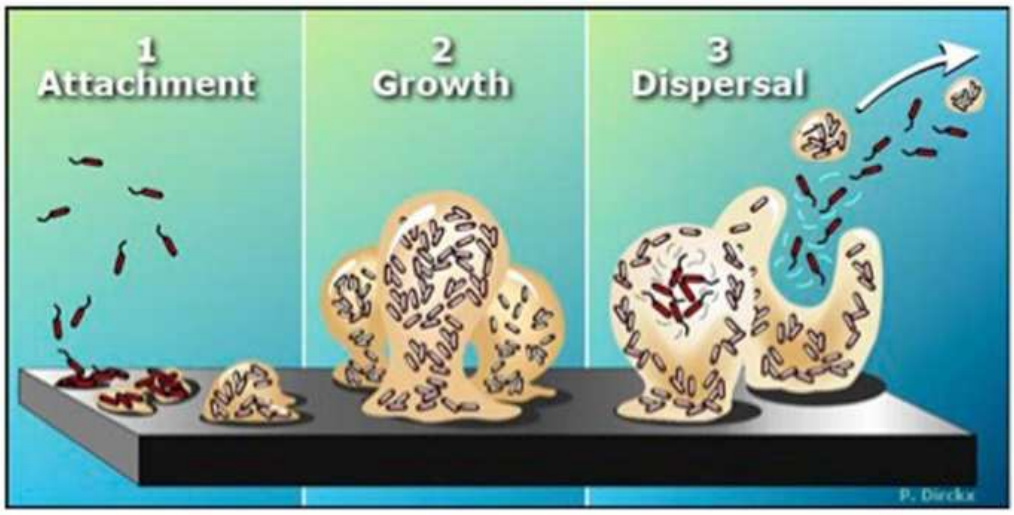

Fig.2. Representation of the biofilm life cycle, with the distinction of the three stages of attachment (1), growth (2) and dispersal (3).

- Attachment: it is the first and most important phase, performed by specialized factors considered virulence determinants, like pili and flagella. *P. aeuginosa* can attach both to biotic and abiotic surfaces and its specific motility stiles, as described before, can mediate the adhesion to substrates characterized by different fluidity. The first contact with the surface is mediated by van der Waals interactions, week and reversible, and is followed by a second stronger adhesion, the real one determining the development of the biofilm structure. Bacterial appendices, including fimbriae, are crucial in this passage; it has beeen reported that bacterial adhesion is more frequent on plastic and other hydrophobic materials than on hydrophilic ones, maybe beacuse of the tendence of these appendices to overcome the small electrostatic interactions occuring in this stage. The first adherent bacterial will act as new layer, favoring the attachment of other cells and concurring to the biofilm growth
- Development: growing phase characterized by intense cellular division and increase of biomass. The first diving bacterial cells originate a "microcolony" (Sriramulu et al., 2005), whose further growth will result in the determination of the biofilm structure. In this phase, cells produce

abundant polysaccharides, that contribute to the maintenance of the all biofilm, often through redundant mechanisms (Colvin et al., 2012). Furthermore, this stage is characterized by a specific genetic expression, promoting the sessile lifestyle and reducing motility.

- Dispersal: in the final stage, usually after 48 h growth, the external layers of bacterial cells are released by the biofilm structure as planktonic cells that have reacquired motility and are able to colonize new surfaces, spreading the biofilm area. The new motile cells can be originated by cell division or by removal of small portions of biofilm. This mechanism is respondent to different stimuli, some of which have been characterized: chemotaxis towards nutrients sources, mediated by flagella; oxygen rate increase; NO rate increase, as metabolic product; presence of chelator agents, surfactans, urea or lysozime; high saline concentrations (Kim and Lee, 2016).

Indeed, *P. aeruginosa* biofilm presents some peculiarities. First of all, the composition of the extracellular matrix is various and at least 3 different kinds of exopolysaccharides, with different functions, have been described (Ghafoor et al., 2011). Alginate is the most famous component, typical of chronic infections isolates; it consists of a polymer, formed by units of glucoronic and mannuronic acid, united by β 1-4 bonds and its main function are to keep the biofilm fluid, to prevent drying and to increase the resistance to both antibiotics and phagocytosis. Although it is not necessary to the initial biofilm production, it has been described as important in forming the most suitable biofilm architecture for *P. aeruginosa*. Indeed, CF chronic isolates, showing alginate overproduction, are characterized by greater dimensions and viscosity, a phenotype called "mucoid", that seems to be functional to the infections maintenance more than induction, since early *P. aeruginosa* isolates result mostly non-mucoid.

The alginate production is due to the operon coding for the *algD-A* genes; this results positively controlled by the σ-factor AlgT that is repressed by the *mucA* gene, a typical mutation hotspot documented in chronic isolates. The mutation accumulation on this target leads to alginate overproduction and to the manifestation of the mucoid phenotype (Wiens et al., 2014).

Iron availability has been described as an important parameter in alginate production (Barton et al., 1996): generally, environmental concentrations lower than 5 μM induce biofilm production, whereas concentrations higher than 10 μM limit it. It has been underlined that even siderophores play a role in alginate expression, since mutations in pyoverdin or pyocyanin genes lead to the loss of iron-dependent regulation and cause a general overexpression of the *algD* gene.

Psl (polysaccharide synthesis locus) is a pentasacchardie containing glucose, mannose and rhamnose. It has a great importance for adhesion both to surfaces, especially plastics and glass, and to other cells, and plays a foundamental role both in the starting phases of biofilm formation and in the detachment of new planktonic cells.

A particular function is the binding of extracellular DNA (eDNA), another main component of *P. aeruginosa* biofilms, contributing to biofilm structure stability and defense against antimicrobial substances. It has been reported that Psl can bind not only the microbial-, but even eukaryotic-eDNA, using it to improve the whole structure fitness (Shiwei et al., 2015).

Pel (pellicle) is the less common and known esoplysaccharide forming *P. aeruginosa* biofilms. Its chemical structure has not been completely identified yet, but it has been described as formed by amino-cationic components, that contribute to eDNA binding and to cationic antibiotics repelling (i.e. aminoglycosides). In general, it is important in the initial stages of biofilm formation, especially in those strains showing a lower psl gene expression and in

environmental strains, where it contributes to the formation of aggregates at the air-liquid interface.

The eDNA is another key component of *P. aeruginosa* biofilms, involved in the formation of cation gradients in all the biofilm structure, in making it more stable and viscous, in the defense against aminoglycoside antibiotics and phagocytosis and in inflammatory processes. It is still uncertain how it is embedded in the biofilm structure: some theses propose an active secretion system, whereas other hypotheses are about bacterial autolysis or secretion by specialized membrane vesicoles. The idea of bacterial autolysis or of auto-recycling of the dead cell DNA seems to match with the definition of biofilm as organized microbial community (Lee and Yoon, 2017).

The whole formation, development and maintenance of biofilm, as well as all the different metabolic and genetic pathways that occur in this growing conditions, are regulated by the cell comunication system known as *quorum sensing* (Lee and Zhang., 2015). This is based on the bacterial production of special molecules, known as autoinducers that are expelled in the external medium; when the bacterial population reaches a certain amount, these molecules act as signals, triggering the genetic expression of specific regulons and modulating the population dynamics. Autoinducers are typically homoserine lactones, but *P. aeruginosa* was described as presenting another class of molecules acting like signals, the alkyl-quinolones, with specific receptors and pathways. Quorum sensing has been described as involved in the coordinated expression of virulence factors and resistance determinants, in the development of mature and differentiated biofilms, in the eDNA and membrane vesicoles release from cells ad in the interaction with the host and other biofilms.

P. aeruginosa presents four main quorum sensing systems, among which three have been well characterized, whereas the last one has been recently described.:

- Las system: based on the N(-3-oxododecanoyl)-homoserine lactone as autoinducer, it is organized like the LuxI/LuxR system, presenting a synthase gene (*lasI*) and a receptor gene (*lasR*). It is a cell concentration-dependent system, involved in the expression of virulence factors (i.e. elastases and alkaline protease), in the biofilm development and in the repression of the small RNA ReaL (Carloni et al., 2017)
- Rhl system: activated by the N-butyryl-homoserine lactone, is composed by the couple synthase/receptor RhlI/RhlR. It is involved in almost every phase of biofilm formation, from the development of the mushroom structures, to the maintenance of water channels and to the detachment of free planktonic cells. Moreover, it regulates the expression of various virulence factors, including rhamnolipids, and pigments (i.e. pyocyanin). An important observation has reported how this system can be stimulated in response to low concentrations of iron and phospate, causing a specific genetic expression in all the bacterial population (Welsh and Blackwell, 2016)
- PQS system: based on the 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) and its precursor 2-heptyl4(1H)-quinolone (HHQ). The latter is produced by the *pqsABCDE* operon and is processed by PqsH, to be secreted out of the cell and to bind the PqsR receptor. This system is involved in the initial biofilm formation, in the LecA lectin expression, in the membrane vesicles formation (Florez et al., 2017) and in eDNA release. As well as the rhl system, it has been described as responsive to iron and phosphate depletion and it is regulated through catabolite repression (Zhang et al., 2013); indeed, the deregulation of this control mechanism could enhance the *P. aeruginosa* fitness during biofilm sustained infections
- The IQS system has been identified in 2013 (Lee et al., 2013) and acts as an integrated system, connecting the las system to the others and being

responsive to phosphate-stress. Strains lacking this system have been reported to be impaired in the other QS systems and to be less virulent

The different described pathways are hierarchically organized in a complex net of communication. The las system represents the main one, being able to respond to bacterial concentration and to activate the other systems. The rhl and pqs systems are connected to las through the IQS system and often regulate each other. Moreover, the environmental stimuli can activate each system indipendently, thus making the microorganism able to efficiently counteract sudden changes in the environment in a coordinate way (Lee and Zhang, 2015).

Given the importance of this system of cell-cell communication, a growing effort has been dedicated to the search for quorum sensing inhibitors, able to impair the genetic pathways controlling *P. aeruginosa* behaviour. So far, some authors have underlined that some known compounds, as azithromycin (Zeng et al., 2016), or molecules affecting other targets (El-Shaer et al., 2016; Rampioni et al., 2017) or plant extracts (Li Y et al., 2017), can represent valid candidates for the development of anti-biofilm therapies.

In the last years, the role of the second messenger c-di-GMP has been reported in bacterial biofilms. The levels of this molecule are controlled by diguanylate cylases and phosphodiesterase enzymes, whose expression is controlled by a wide range of environmental stimuli and regulator systems. In particular, c-di-GMP seems to be tightly controlled by the Gac/Rsm system and its action is often reported as antagonist to the RsmA protein's one; c-di-GMP levels result, infact, higher in biofilm growh and to be correlated with chronic infections, whereas RsmA is responsible for the regulation of various factors involved in acute infections (Moscoso et al. 2011). As far as concerns c-di-GMP regulator role, it is involved in the control of *P. aeruginosa* motility, adhesion, exopolysaccharide production and even efflux activity.

Biofilm lifestyle represents a winning and feared strategy for bacteria for many reasons. It allows them to colonize large surfaces and, most importantly, almost every kind of environment, several eukaryotic hosts and even artificial medical implantations. The biofilm structure itself assures a strong adhesion to the substrate and resistance to mechanical stress, making its detachment very complicated.

The thickness and viscousity of the matrix act as a real barrier to a wide range of molecules and antimicrobial agents: both in environmental and clinical settings, *P. aeruginosa* disinfection is hampered by its biofilm, since a lot or detergent cannot penetrate the exopolysaccharide matrix, as well as many antibiotics, that often reach their specific target in too low concentration to exhibit a positive effect. Moreover, biofilm limits the phagocytosis performed by macrophages and contributes to immune system cell death through the expression of specific virulence factors such as rhamnolipids (Alhede et al., 2014).

Biofilm cells are even characterized by a specific gene expression that results different from their planktonic counterpart, involving the production of specific molecules, including virulence factors, the modulation of messengers levels and the activation of specific metabolic patterns. Moreover, antibiotic resistance is potentiated in sessile cells (Soto, 2013), mainly through the decrease of the growth rate and metabolism, making the cells less susceptible to antibiotic action, and the overexpression of specific resistance determinants. The most common are represented by efflux pumps that play either a physiological role in biofilm development itself and are potentiated in their activity of expulsion of antibiotics out of the cells when bacteria grow as biofilm. Some genes are specifically expressed during biofilm growth; as example case, the *ndvB* gene has been described as one of the main determinants of aminoglycoside resistance of *P. aeruginosa* biofilms (Beaudoin et al., 2012). This gene codes for a glycosiltransferase responsible for the production of cyclic glucans, localized in the bacterial periplasm, and controls the expression of other genes related to

ethanol oxidation. The cyclic glucans represent a non-specific resistance mechanism, since they can sequester and inactivate aminoglicosydes, in particular tobramycin, before they reach their cytoplasmic target.

Biofilms are crowded microbial communities, belonging either to the same or to different species. They represent the perfect background for microbial interactions. The coinfection of CF lung by bacteria of different species, especially *Staphylococcus aureus* and *P. aeruginosa*, is quite common and recently a new *P. aeruginosa* tobramycin resistance mechanism, based on *S. aureus* pre-infection, followed by specific interactions between the two bacterial species, has been described (Beaudoin et al., 2017). Moreover, the growing in the exopolysaccaride matrix favors close contacts between bacterial cells which can result inc Horizontal Gene Transfer (HGT) events, both intra-species (Li G et al., 2017) and inter-species (Tanner et al., 2017).

Finally, specialized phenotypes and bacterial populations have been described in biofilms. These bacterial communities are adapted to this specific microenvironment and each one seems to play a specific role in the infections dynamics (Sherrad et al., 2014). *P. aeruginosa* biofilms are often present in the infection development. They have been described in wound infections (Hunt et al., 2017), urinary tract ones (Newman et al., 2017), ocular ones (El-Ganiny et al., 2017), bloodstream ones (Lebeaux et al., 2014) and, especially in CF, pulmonary infections.

2.5 Antibiotic resistance

P. aeruginosa is characterized by a wide arsenal of antibiotic resistance mechanisms that result responsible for the failure of various applied antibiotic therapies.

It is, indeed, a common feature of *P. aeruginosa*-caused infections, particularly in CF chronic lung infections, the involvement of MDR strains and even EXtensively Drug Resistant (XDR) strains. The only antibiotic that still appears to contrast *P. aeruginosa*, or at least to select the lowest number of resistant clones, is colistin. However, its systemic use, due to the absence of inhaled formulations, results in a toxicity rate which led to limit its use for routine anti-*P. aeruginosa* treatment (Koerner-Rettberg and Ballmann, 2014).

P. aeruginosa antibiotic resistance relies on both intrinsic and acquired determinants. Among the formers, the outer membrane itself represents an efficient barrier to antimicrobials penetration. This is mainly due to the small volume of the main porins and to the low number of larger porins, limiting antibiotic diffusion. In 2003, Wang et al. described a two component-system able to up-regulated *P. aeruginosa* membrane permeability, resulting in a greater antibiotic, especially aminoglycoside, susceptibility.

Efflux pumps are additional important determinants of intrinsic resistance. They are usually divided in 5 families (Li and Nikaido, 2009):

- ATP-binding cassette family (ABC);
- Major facilitator superfamily (MFS);
- Multidrug and toxic compound extrusion family (MATE)
- Small multidrug resistant protein family (SMR);
- Resistance-Nodulation Division family (RND).

The last one is probably the most represented in *P. aeruginosa*; at least four complexes belonging to this cluster have been described (Terzi et al., 2014) and each one can act as a multidrug expelling complex. They all share the same protein organization (Ruggerone et al., 2013), shown in Fig. 3: an inner membrane trasporter, constituting the motor protein of all the system, recruiting and sequestering the antibiotic molecule from the bacterial cytoplasm/periplasm; an outer membrane protein, forming the channel that allows the extrusion of the toxic compound out of the cell; a periplasm protein acting as a junction and allowing the all mechanism to be functional and active.

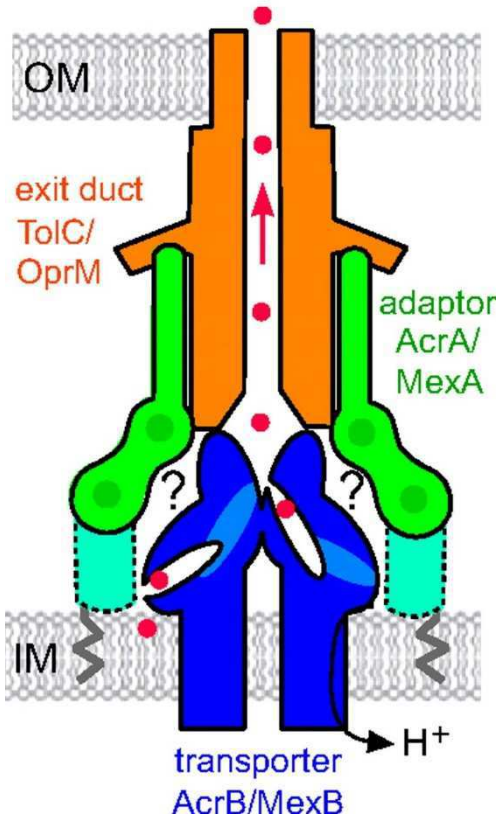

Fig. 3. Schematic representation of a RND efflux pump, reporting the AcrAB-TolC and MexAB-OprM models of *E. coli* and *P. aeruginosa* respectively.

Efflux pumps are involved in the so called "adaptive resistance", characterized by a resistance phenotype in response to a specific environmental stimulus; even in this case, aminoglycosides are the antibiotics mainly involved (Hocquet et al., 2003). However, the regulatory genes responsible for the modulation of their

expression represent mutation hotspots; in chronic infections they are often inactivated, resulting in an enhanced efflux, compared to that exhibited by the reference strains like *P. aeruginosa* PAO1. Another typical feature is the wide range of substrates they can expel and, most of all, the redundancy of target-specificity, i.e. the same antibiotic can be expelled by more than a single efflux pump, making quite difficult to contrast this mechanism of antibiotic resistance.

As well as other Gram-negative rods and enteric bacteria, *P. aeruginosa* produces a chromosomial β-lactamase, encoded by the *ampC* gene, which contributes to β-lactams resistance (Pérez-Gallego et al., 2016). In particular, this enzyme overexpression seems to be linked to the lack or mutation of many effectors involved in cell wall recycling pathways, especially the N-acetyl-anhydromuramyl-l-alanine amidase encoded by the *ampD* gene. This enzyme, responsible for degrading peptidoglycan catabolites during bacterial growth, suppress the *ampC* expression. In its absence or in presence of high β-lactam concentrations, peptidoglycan catabolites tend to accumulate in the bacterial cytoplasm, triggering *ampC* induction. Recently the main protein regulating the *ampC* expression, AmpR, has been described as one key regulator gene not only in antibiotic resistance, but even in other *P. aeruginosa* physiological, including virulence, pathways (Balasubramanian et al., 2015). Interestingly, AmpR seems to negatively regulate alternative resistance mechanisms, such as efflux pumps, modulating the expression of their regulatory genes, the OxaB carbapenemase, another chromosomal- encoded determinant, and the production of the Aph class of the aminoglycosides modifying enzymes. AmpR is also involved in the quorum sensing system, positively regulating the expression of the regulator component of every quorum sensing system, the iron uptake and the expression of several virulence factors, including elastases and pyocyanin. Finally, it is involved in the bacterial stress response, since it positively controls the RpoS σ-factor, the gene mainly involved in the control of *P. aeruginosa* response to various stimuli.

P. aeruginosa is considered one of the most versatile bacteria, endowed with a large chromosome containing the largest proportion of regulatory genes observed in a sequenced genome; moreover, it results particularly prone to changes by acquiring new genetic informations, following both the mutations of intrinsic chromosomal genes and the acquisition of exogenous mobile genetic elements from other strains.

The mutations involving antimicrobials targets are often easily selected and maintained. This is the case of the resistance to fluoroquinolones due to the mutation of the genes encoding the enzymes DNA gyrase and/or topoisomerase IV, which is maybe the second most diffused antibiotic resistance mechanism. These mutations occur in specific DNA sequences, named Quinolone-Resistance Determining Regions (QRDR), i.e. those coding the subunits A and B of the DNA gyrase and the parC and parE of the topoisomerase IV. The study performed by Bruchmann et al., (2013) underlines that mutations in these regions are not only responsible for the resistance insurgence, but that they can even accumulate in a coordinated way, increasing the antibiotic resistance of the strain. The association of mutational events and the overexpression of efflux pumps is responsible of the ciprofloxacin-resistance shown by most CF *P. aeruginosa* isolates.

The acquisition of exogenous DNA through HGT events, besides causing the spread of antibiotic resistance among *P. aeruginosa* strains, plays a critical role in the emergence of virulent strains as well as in their adaptation to a particular environmental niche; this is particularly evident in Cf strains (Qiu et al., 2009), where the acquisition of pathogenicity islands is responsible for the infection development. Among the DNA modification events that shape *P. aeruginosa* accessory genome, HGT is considered the main one. In particular, *P. aeruginosa* has been described to be engaged in conjugation and transduction events, whereas not so much evidence of natural transformation events has been reported (Kung et al., 2010). Moreover, it has been described that several *P.*

aeruginosa strains carry combinations of acquired genes, showing no homology with the *P. aeruginosa* genome, highlighting the great contribution of interspecies HGT to the genetic variability of this opportunistic pathogen (Pohl et al., 2014). The accessory genome elements of *P. aeruginosa* can be splitted in 4 categories (Kung et al., 2010): integrative and conjugative elements, integrons, prophages and phage-like elements, and transposons/insertion sequences. Plasmids further contribute to the *P. aeruginosa* genomic variability. Investigations on antibiotic resistance mechanisms and the detection of more and more new resistance genes has led to the fine characterization of these elements. The aminoglycosides are perhaps the antibiotic class presenting the highest number of resistance determinants encoded by extrachromosomial genetic elements. Infact, excluding the MexXY-OprM efflux pump and the cyclic glucans in the periplasmic space, the resistance against these antibiotics is mainly mediated by the production of Aminoglycosides Modifying Enzymes (AMEs) (Poole, 2005 B), encoded by mobile elements (mainly integrons) also encoding for additional antibiotics resistance determinants. A typical example is constituted by the gene *ant(2")-Ia*, encoding for an adenylating enzyme, usually carried by multiresistant class I integron (Kim et al., 2008; Mózes et al., 2014).

Also the 16S rRNA methylase encoded by the gene *rmtA* is a resistant determinant acquired by HGT. This enzyme, first characterized in 2003 (Yokoyama et al., 2003), showed a high similarity with the methylases produced by other microorganisms, suggesting a "non-Pseudomonas" origin. One year later, (Yamane et al., 2004) it was indeed described as located in a transposon.

Beta-lactamases are another important class of resistance determinants often found in *P. aeruginosa*. They may be present in different isoforms and are often organized in peculiar gene cassettes within integrons (Juan et al., 2008; Jeong et al., 2009). They can be easily transferred by HGT between different *P. aeruginosa* strains (Kitao et al., 2009) and also between strains of *P. aeruginosa* and strains belonging to different bacterial species (Libisch et al., 2008).

The maintenance of this large panel of resistance genes causes some fitness costs to this pathogen (San Millan et al., 2015), but this is compensated by the positive selection they undergon during chronic infections, when bacteria are exposed to several stress conditions, including the antibiotic treatment. Resistant genes are indeed involved in the occurrence of endemic infections (Mózes et al., 2014) and it is not surprinsing that XDR strains are isolated in the nosocomial envinronment (Oliver et al., 2015). Furthermore, a recent work described how fluctuant concentrations of antibiotics can promote the antibiotic resistance acquisition without fitness costs for *P. aeruginosa* (Melnyk et al., 2017).

To cope with this complex and wide range of resistance mechanisms, the most adopted strategy is the use of association of antibiotic, in order to simultaneously attack different bacterial targets and to avoid the development of resistance. In the particular case of CF, *P. aeruginosa* lung infection treatment includes the association of orally administered ciprofloxacin and inhaled antibiotics, mainly colistin and tobramycin (Schelstraete et al., 2013); some studies have even reported the use of miscellaneous combinations, characterized by the addiction of ceftazidime, ticarcillin or imipenem. In the latest years, the main *P. aeruginosa* eradication therapy is based on inhaled antibiotics (i.e. tobramycin, aztreonam and colistin), whose combinations, even with additional molecules, are often administered to counteract MDR *P. aeruginosa* infections; some cases report that the use of ciprofloxacin is often unable to completely eradicate the pathogen (Hayes et al., 2011), so it is often sostituted by intra-venous β-lactams. More recently, the association fosfomycin/tobramycin association has been proved to be efficient in coping *P. aeruginosa* infections and it is planned to undergo Phase III of clinical trial (Antoniu, 2015).

In conclusion, despite the several attempts to control and eradicate *P. aeruginosa* infections, this opportunistic pathogen still represents a worldwide health threat in particular as regards CF infections. At present, the clinical microbiological reasearch is now focusing on the identifications of new

strategies to treat the infections caused by this microorganism. The most promising options include:

- The phage therapy, with the use of cocktails of phages as efficient and not immunogenic treatment (Shiley et al., 2017)
- The use of new formulations of natural compounds and extracts already known for their antimicrobial activities in new formulations (Nobakht et al., 2017)
- The use of natural pumps inhibitors (EPIs) to be included as adjuvants in already existing therapeutic protocols (Aghayan et al., 2017)
- The development of new drug delivery strategies and new substrates to enhance antibiotic action (Craparo et al., 2017)

3. Pulmonary infections sustained by *Pseudomonas aeruginosa* in cystic fibrosis

3.1 Cystic Fibrosis

3.1.1 Genetic disorder

Cystic Fibrosis is a genetic autosomic inherited pathology, caused by the mutation of the *cftr* gene, localized in the long arm of the human chromosome 7 (7q31.2) and responsible for the production of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein (Rommens et al., 1989). This is a cytoplasmic membrane protein that regulates the chloride ions transport to the extracellular environment in epithelial tissues, contributing to the saline balance and, more in general, to the tissue homeostasis maintenance. It belongs to the ABC transporter family and it is the unique representative acting directly as an ion channel (Meng et al., 2017); it is composed by two nucleotide binding domains and two transmembrane domains, that form the channel pore, separated by a regulatory domain known as R-region. This last portion presents various phosphorilation sites, targeted by the protein kinase A, that responds to intracellular levels of ATP. According to the model described by Vergani et al. (2005), the ATP intracellular accumulation triggers the phosporilation of the R-region, that loses its allosteric role and allows the dimerization of the nucleotide binding domains; this conformational changes allow the opening of the channel and the free exit of chloride ions from the cell. According to a second model (Bozoky et al., 2013), the structural changes of the channel are due to the interaction of the R-region with the C-terminal portion of the CFTR protein itself, attributing to this region the role of global regulator of the entire process.

The channel is sinthesized as chaperone complexes in the endoplasmic reticulum, then a variable portion between 25% and 50% of the total produced proteins are released from the chaperones and trasferred to the Golgi apparatus; here the protein is subjected to further maturation processes and is finally

transferred to the cytoplasmic membrane, where the phosphorilation of the R-region allows its correct function (Ward et al., 1995). A large part of the translated protein is still retained in the endoplasmic reticulum and subsequently degradated by the ubiquitin-proteasome pathway.

The *cftr* mutations alter the normal folding process of the channel or the maturation pathway leading to its localization in the cell membrane. Among the various described mutations, the most frequent is F508del (Amaral, 2004), causing the deletion of a phenylalanine in position 508; this leads to a misfolded protein, that tends to accumulate in the endoplasmic reticulum, to be degraded by proteasomes (Ward et al., 1995), and to reduce the channel activity by an effect called "gating defect" (Sondo et al., 2017). The final outcome is the impairing of the chloride ions trasport through the cell membrane. Furthermore, this mutation has been described to induce the formation of aggresomes and the upregulation of reactive oxygen species, increasing the cell damage and inhibiting autophagy processes (Luciani et al., 2010). Other related effects comprehend the downregulation of other ion transporters, whose activity is coordinated with CFTR (Bertrand et al., 2017), a minor stability of the cell transcript with the subsequent activity of proinflammatory RNA-binding proteins (Biswas et al., 2017), impairment in actin and tight junctions organization (Castellani et al., 2017) and, in human macrophage, a defective pathogen clearence (Ferrari et al., 2017).

Thus, the detection of this particular mutation has been proposed as first screening and prediction of clinical outcomes that have been proven to be connected with the pathology genotype, being more severe in the homozygous condition (Marson et al., 2013).

The compensation of the *cftr* gene mutation has now become the target of new therapeutic approaches in order to attenuate or to resolve the CF basic genetic defect. The most known treatment option is the combination of CFTR corrector

(i.e. Lumacaftor), that enhances the percentage of CFTR protein transferred to the cell membrane, and a potentiator (i.e. Ivacaftor), that reduces the gating defect (Deeks, 2016). This association is generally subministered to patients older than 12 years, but a recent study has evidenced that even younger subjects can easily tolerate the therapy (Milla et al., 2017).

Furthermore, new studies have been focused on the identification of alternative targets that can modulate CFTR deficiency, and two promising hits have been selected.

The protein kinase CK2 has been described as a molecule contributing to CFTR premature degradation (Venerando et al., 2014). This protein recognizes a specific motive that is present in the position 511 in CFTR protein; however, it has been demonstrated that the CF channel can be phosphorilated only if the residue 512 is previously modified by a tyrosin kinase, underlining a complex phosphorilation pattern, now object of reasearch in order to delay CFTR degradation by the cellular proteasomes.

Another promising target is represented by the ubiquitin ligase Rnf5 (Tomati et al., 2015), acting in the early stages of CFTR biosynthesis and promoting its degradation. This gene knockout in a murine model has demonstrated to improve intestinal absorption and to reduce weight loss and fecal excretion of biliar acids. Moreover, *rnf5* expression has resulted different in CF respiratory epithelial cells compared to healthy subjects, indicating a possible specific target for an attenuating treatment.

Since till now no therapy has proved to be resolutive in the CF defect correction, the main treatment option is currently based on the combination of the corrective therapy with other adjuvant ones, in order to target different hits of the same biosynthetic pathway.

3.1.2 Symptoms and manifestations

CF promotes a severe clinical condition, affecting several system of human body. The lack of the CFTR channel is responsible for the impairment of the saline balance in epithelial tissues, leading, as final result, to the production and accumulation of a stick and viscous mucus on the tissue surface (O'Sullivan and Freedman, 2009). This is responsible for the pathologic aspects of CF, since it creates obstructions in the epithelia and it blocks the mucociliary movement aimed to perform the microbial clearence and to prevent infections by pathogens.

The respiratory system is the body district most affected by CF (Cohen-Cymberknoh et al., 2013), suffering from a continous and self-perpetuating neutrophilic lung inflammation, induced by the mucus obstruction of the airways. The clinical outcome is various and complicated. Pulmonary exacerbations are very common and can occur at every age; they are characterized by cough, shortness of breath, fever and weight loss, and greatly affect lung function (Anstead et al., 2014). They are typically correlated with the presence of bacterial infections, representing the main cause of morbidity and mortality for CF patients, since microorganisms can both cause tissue damage by secreting extracellular enzymes and induce a strong immune response.

Pulmonary function itself is often described and controlled by referring to spirometric parameters like the average forced expiratory volume in 1 s (FEV_1), the forced vital capacity (FVC) and the forced expiratory flow at mid-lung volume (FEF_{25-75}), considered meaningful in the prediction of CF pulmonary outcomes (Wagener et al., 2015).

CF lung often presents various signs of complication (NG et al., 2014). Bronchiectatis is the typical hallmark of CF, presenting an abnormal and permanent airways expansion that in the worst cases can develop into a cystic form; its presence hampers further CF complications identifications, since

comorbid status cannot be clearly evidenced during radiography exams. Other clinical manifestations include pneumothorax, a perforation of the pleural space due to the air pressure in the alveoli caused by mucus accumulation, atelectasis and lobar collapse. The final outcome is a long decline of lung fucntion, ending in respiratory insufficiency and lung collapse. In the advanced phase of the pathology, chronic hypoxia and subsequent pulmonary hypertension may occur as well. At the end-stage and/or severe conditions, lung transplantation is performed in order to increase the patients median survival and, indeed, CF respiratory failure represents the third most common cause of performance of this procedure (Dorgan and Hadjiliadis, 2014).

In some cases, plugging and distension of the submucosal gland ducts have been described, suggesting even a prenatally modification of the CF lung (Cohen-Cymberknoh et al., 2013). It has been evidenced that an early treatment of respiratory symptoms in young patients is efficient in improving lung function and health conditions in the following years, suggesting that the early pathology stage is the best time to treat the respiratory defect (Grasemann and Ratjen, 2013).

Other than the respiratory system, CF affects multiple organs, including pancreas, liver and intestine (Fraquelli et al., 2016), occurring in 80-90% of CF patients and causing intestinal obstruction syndrome, liver disease, cirrhosis and biliary tree abnormalities. It has even been associated with diabetes, which is more influent on nutritional conditions rather than cardiovascular pathologies and is reported in almost 50% of adult CF patients (O'Shea and O'Connell, 2014).

Finally, it has been reported that all male and almost 50% of female CF patients suffer from infertility issues: in male individuals, CFTR mutation is associated with azoospermia, absence of the vas deferens and reduced sperm functionality,

whereas in female ones the poor healthy and nutritional conditions severely impact on fertility (Jones and Walshaw, 2015).

Considering all these manifestations, the early CF diagnosis and the subsequent patient monitoring appear to be pivotal as best tool to prevent clinical complications and to assure good health conditions to CF patients.

3.1.3 Diagnosis

CF is often diagnosed in childhood, few weeks after birth, by newborn screening, in order to reduce childhood morbidity, delay the pathology mortality and provide genetic information to families (Massie and Gillam, 2014). The first screening tests were based on the immunoreaction to trypsin detected in infant blood spots, performed at 2-4 weeks of age and repeated at 4-6 weeks if an elevated immunoreactivity of trypsin was detected. After the identification in 1989 of the *cftr* gene as responsible for the pathology, a genetic screening was added, allowing the gene mutations identification. The main routinely performed test remains the sweat test, considered the gold standard in CF diagnosis and allowing the identification of over 1900 genetic mutations (Mattar et al., 2014). The classic procedure, described by Gibson and Cooke (1959), consists on the measurement of the sweat mass and chloride analysis after skin stimulation by iontophoresis. According to the UK Guidelines for the interpretation of the sweat test (Green et al., 2007), a sweat chloride concentration >60 mmol/L supports the CF diagnosis, especially if combined with a suspect phenotype; an intermediate value 40-60 mmol/L is considerated suggestive of CF, wherease values <40 mmol/L are typical of healthy individuals.

Regarding the conductivity measurements, only values >90 mmol/L supports the CF diagnosis, but they are not considered enough to indicate a reliable diagnosis.

In the latest years, the importance of predicting CF insurgence has become pivotal, and an increasing effort has been dedicated to develop and diffuse carrier screenings as a complementary tool, in order to provide families with major informations about the incidence of this pathology (Castellani and Massie, 2014; Mosconi et al., 2015).

3.2 Bacterial infections

CF pathologic condition is made even more severe since comorbid with bacterial infections. The mucus produced by epithelial cells indeed blocks the mucociliary movement on their apical side, preventing the clearence of microorganisms that can adhere to them; moreover, the mucus itself represents a favoring substrate for bacterial adhesion and proliferation (Döring et al., 2011). Indeed, the tissue damage typical of CF is due mainly to the action of bacterial toxins and exoenzymes and to the inflammatory response the immune system acts to counteract infections (Nichols and Chmiel, 2015). The CF respiratory system really represents a perfect model to study bacterial pathology, interactions and dynamics, since the CF microbiome evolves during the patient's life, showing the predominance of different bacterial species in determined infection stages, the adaptation to the lung environment, a different genetic expression profile and the development of persistence strategies to survive antibiotic treatment.

CF patients are colonized by a wide range of microorganisms, as reported in Fig. 4; among bacteria, *S. aureus* represents the first colonizer and the most isolated in early infections in CF patients (Goss and Muhlebach, 2011). It has been estimated that 30-50% of healthy patients intermittently or chronically harbor *S. aureus* in their nasal carriage, constituting a continuous reservoir of respiratory infections. This pathogen is generally aerobic, but even an anaerobic and

biofilm-forming life style has been described in CF, associated with chronic infections.

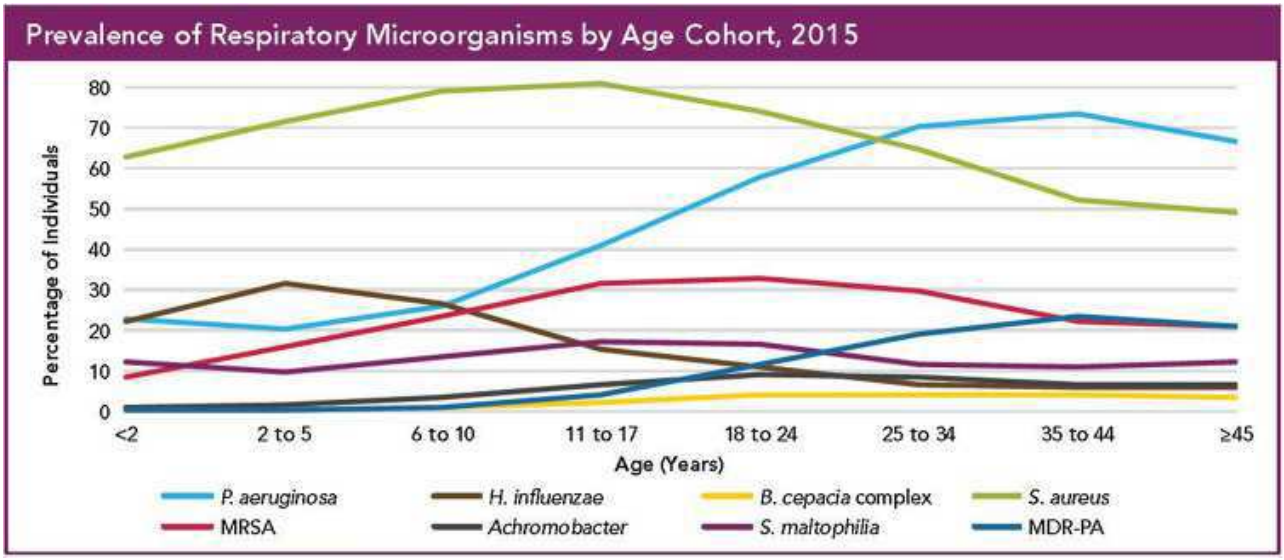

Fig.4. Prevalence of infective microorganisms in CF patients. The percentage of individuals reporting a specific pathogen infection is correlated with the patients age; the results are referred to a survey performed in 2015.

Methicillin-Resistant *S. aureus* (MRSA) represents a great health-treat for CF patients, and actually it is being recognized as a growing problem, comparable to *P. aeruginosa* infections. Since it plays a great role in pulmonary exacerbations and it is well characterized by a wide range of antibiotic resistance mechanisms, staphylococcal isolates are subjected to typing of both the bacterial cells, by Pulsed Field Gel Electrophoresis (PFGE) and MultiLocus Sequence Typing (MLST), and the Staphylococcal Casette Chromosome (SCC)*mec* types. Till now, 8 types of (SCC)*mec* have been discribed; types I, IV and V are characterized by the exclusive resistance to β-lactams resistance, whereas types II and III usually carry even additional antibiotic resistance genes.

S. aureus and specifically MRSA antibiotic treatment in CF is performed through the use of different antibiotic classes (Fusco et al., 2015). Vancomycin and linezolid are considered first-line options for infections treatment and are often coupled with other molecules, such as doxycycline or minocycline and

trimethoprim/sulfamethoxazole, providing overlapping protection towards other infecting bacteria. New antibiotics like ceftaroline and ceftobiprole seem to show a good potential in staphylococcal infection treatment, whereas clindamycin has been abandoned, since showing a great rate of resistance.

A very interesting aspect is the interactions between *S. aureus* and other CF pathogens, in particular *P. aeruginosa.* The two bacteria often coexist in particular niches of the CF lung environment and have been demonstrated to specifically interact (Baldan et al., 2014). In particular, it has been observed that small *P. aeruginosa* molecules, like the messengers of PQS system and other exoproducts, can stimulate biofilm production in *S. aureus* and CF lung environment promotes the selection of adaptive and interacting strains (Fugère et al., 2014). Moreover, the co-culture of these two pathogens have showed to induce the formation of Small Colony Variants (SCVs) in *S. aureus*, favoring its persistence (Filkins et al., 2015).

However, in CF lung, *S. aureus* is not the only infective pathogen other than *P. aeruginosa*. *Haemophylus influenzae* has been described as one of the first colonizers as well (Renders et al., 2001), and some airway colonizers have been identified as emerging pathogens, like *Stenotrophomonas maltophila* and *Achromobacter xylosoxidans* (Ciofu et al., 2013). Interestingly, a growing interest is now dedicated to the non-tubercolous mycobacteria, especially to *Mycobacterium abscessus*, that seems to play a great role in CF infections (Gilligan, 2014). Finally, new evidences are reporting of a complex respiratory microbiome that is described to be more variable in the lower than in the upper airways, and seems to include even strict anaerobe species, mostly belonging to the genus *Prevotella* (Surette, 2014).

CF patients are even exposed to the infection by a wide variety of fungi. The colonization by these microorganisms colonization seems to be facilitated by different factors, including the immune system disorders of CF patients, the

prolonged antibiotic therapies and the use of corticosteroids (Pihet et al., 2009). The most commonly isolated species are *Aspergillus fumigatus* (Moss, 2015), *Scedosporium apiospermum* and *Candida albicans* as major yeast (Chotirmall and McElvaney, 2014), but even less common species have been described as able to chronically infect the lower airways and some of them seem to be exclusively isolated in the CF context (Pihet et al., 2009).

3.3 *P. aeruginosa* infections

3.3.1 Early infections

P. aeruginosa remains the apex pathogen and the most feared one in the CF microbiology. With growing age, almost all patients are infected by this bacterium, and the infection usually becomes chronic, ultimately leading to death by pulmonary insufficiency.

The first infection can be acquired even by 3 years old children, without showing evident symptoms. This is a critical period, since it has been recognized that early colonization is the time that offers the best chances for *P. aeruginosa* eradication by antibiotic treatment (Langton Hewer and Smyth, 2014). The importance of an early and efficient diagnosis is pivotal to detect and monitor the pathogen presence; different studies and clinical experiences have tried to identify useful markers in order to detect early *P. aeruginosa* colonization. The use of serology has proved to be reliable in the monitoring of early infected patients (Dolce et al., 2014) since, in this stage, a specific immune response, given by antibodies targeting *P. aeruginosa* typical exoenzymes, was obtained. However, the fail in predicting *P. aeruginosa* infections and the overlapping response with healthy subjects limit its use from main diagnostic tool to a confirming technique for the classical culture assays (Taccetti and Sly, 2014). Another study evidenced that *in vitro P. aeruginosa* phenotypic profiles can be associated with different infection stages (Mayer-Hamblett et al., 2014); in

particular, in the shift from early to intermittent and chronic infections, pyoverdine production and mucoid colonies show an increment, whereas protease production and twiching motility seem to be reduced.

A geographical investigation about the risk factors for *P. aeruginosa* infections did not show particular differences between regional and metropolitan children, and most of all, no evidence of cross-infection was detected (Ranganathan et al., 2013). This result sheds light in two main features of *P. aeruginosa* infections: the main source of infection is environmental and bacterial colonization is acquired by patients in the community; generally, there is not a specific clone spreading among patients, but each individual presents a particular strain.

Nevertheless, cases of epidemic strains, representing a great concern, have been reported. The most famous is the *P. aeruginosa* Liverpool epidemic strain (LES) (Fothergill et al., 2012), characterized by ceftazidime resistance, the ability to produce a superinfection in previously infected patients, to survive for prolonged periods on dry surfaces and, for a short period, even in air samples. There is a general consensus that LES infected patients face a worse prognosis compared to patients carrying other *P. aeruginosa* strains. More in general, transmissible clones negatively impact on patients condition, leading to lung transplantation as ultimate resource (Srour et al., 2015). Evidences of *P. aeruginosa* epidemic clones have been reported even in Australia (O'Carroll et al., 2004) and Ireland (Logan et al., 2012), and a novel clone termed "prairie epidemic strain" (PES), characterized by a wide and allarming antibiotic resistance, has been described in Canada (Parkins et al., 2014).

Patients showing an early *P. aeruginosa* infection are subjected to an aggressive antibiotic therapy, in order to achieve a fast pathogen eradication. The most adopted treatment is based on the assumption of tobramycin inhaled solutions, since it has proved to efficiently reduced the pathogen abundance in the lung environment and to maintain it free from the pathogen colonization over 1-2

months period (Ratjen et al., 2010). Unfortunately, the most common outcome is the survival of *P. aeruginosa*, that subsequently establishes an intermittent and then chronic infection.

3.3.2 Infection diagnosis

P. aeruginosa pulmonary infection diagnosis is routinely performed by culture-dependent identification from CF sputum samples. This type of sample is high representative of the colonization of the lower airways, resulting more sensitive than the nasopharyngeal swab and less invasive than the bronchoalveolar wash. (Burns and Rolain, 2014). A comparison of these three kinds of sample for *P. aeruginosa* detection revelead indeed that a negative result obtained from a throat swab is not predictive enough to exclude the pathogen presence (Seidler et al., 2016).

The sputum sample is usually homogenized with a solubilizing agent, in order to reduce the mucus viscosity and to reliably culture the various microorganisms (Burns and Rolain, 2014). Cultures are performed on rich media and, in the case of *P. aeruginosa*, on MC agar. Despite the availability of *Pseudomonas* selective media, they are not usually recommended as a diagnostic tool, since they could inhibit *P. aeruginosa* growth; this is a crucial point, since this pathogen, especially the mucoid variant, shows per sé a slow growth, and it requires rich media to be correctly cultured.

Once the pathogen is isolated in pure culture, rapid biochemical tests (i.e. catalase and oxidase) can help the identification that is usually achieved through biochemical tests batteries or Maldi-Tof mass spectometry. However, since *P. aeruginosa* often shows phenotypic variability and slow growth, these methods have been questioned about their reliability, also considering the limits of the information databases they rely on.

Molecular PCR-based method are arising the interest of the clinicians as diagnostic tool, but at present their use is still limited compared to cultural assays.

3.3.3 Adaptation to the lung environment

Once *P. aeruginosa* colonizes the CF lung environment, it starts and adaptation process causing the modification of many of its genetic and biochemical pathways and allowing its survailance and persistence within the host, as reported in Fig. 5.

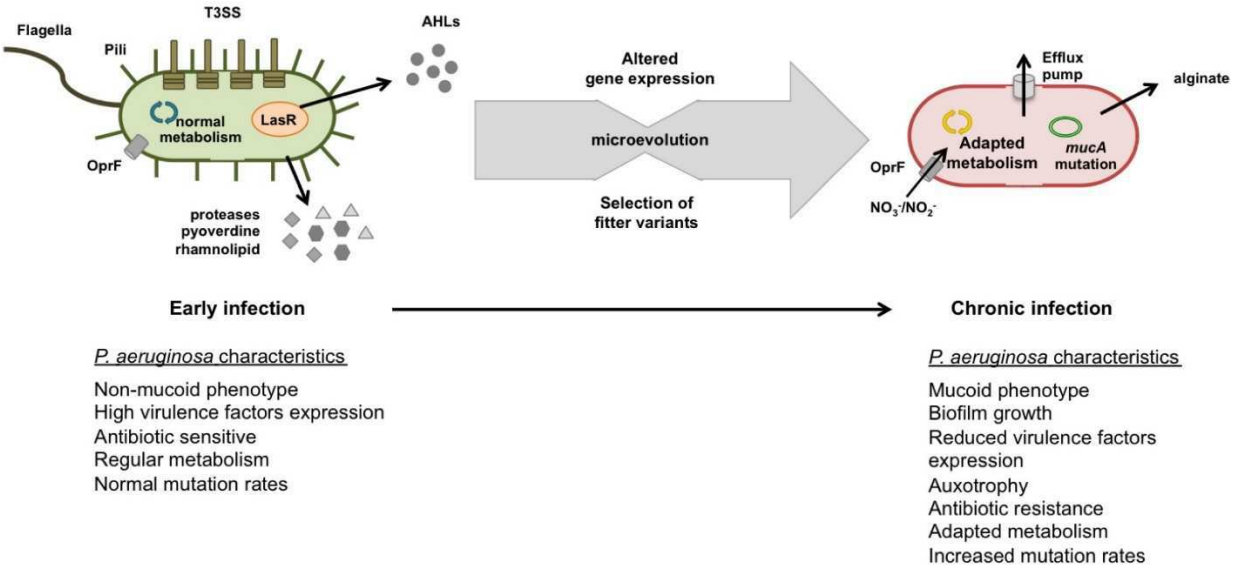

Fig. 5. Adaptive evolution of *P. aeruginosa* to the CF lung environment. The main metabolic and genic expression changes are reported

This infection stage always coincides with the biofilm formation and leads to the induction of a recurrent infection. This modification is possible thanks to the wide and plastic *P. aeruginosa* genome, rich of regulatory sequencences, and to its accessory genetic information, easily transmitted among different strains during infection (Döring et al., 2011). From a phenotypic point of view, the adaptation to the lung environment causes the emergence of particular phenotypes; the mucoid one is absolutely the most common, presenting an

alginate overproduction and the formation of a viscous capsule protecting the bacterial cell. This change of genetic expression is often due to mutations in the regulatory genes affecting the exopolysaccharide synthesis, but it has to be reported that mucoid *P. aeruginosa* strains, efficiently selected especially during a chronic infection, can subsequently revert to the non-mucoid phenotype. Another *P. aeruginosa* colony variant isolated during chronic infections is represented by the SCVs, i.e. smaller colonies, characterized by a slower growth compared to those observed in the early stage of infection.

A particular interest is growing about the "rugose SCVs" phenotype, characterized by enhanced biofilm formation, enhanced psl and pel exopolysaccharide secretion, lower motility, a defect in growth on some amino acid and the use of some intermediates of the tricarboxylic acid cycle as sole carbon sources (Starkey et al., 2009). Other phenotypic changes include the loss of motility and pigmentation in the majority of strains recovered during intermittent and chronic infections. Moreover, from a structural point of view, the lipopolysaccharide loses the O antigen and the lipid portion is modified, resulting in antibiotic resistance to aminoglycosides and other positively charged compounds.

Biochemically, *P. aeruginosa* shows a general metabolic reduction and the development of different metabolic pathways characterized by the respiratory reduction and by the modification of the primary carbon source adopted by bacterial cells, specifically amino acids, carbohydrates and carboxylates. Interestingly, this variability is not related to the infection stage (i.e. early, intermittent or cronic) or to the *P. aeruginosa* phenotype (i.e. mucoid or non-mucoid) but to the particular environmental niche colonized by a specific subpopulation. As an example, some differences, can be noted between *P. aeruginosa* isolates recovered from patients' lungs or sinus (Jørgensen et al., 2015).

A general adaptive strategy adopted by this pathogen is the reduction of the expression of virulence factors and a major commitment to express resistance determinants (Döring et al., 2011). This has the double effect of inhibiting the immune system recognition of the bacterial presence, as well as its response, and to counteract the constant exposure to antibiotics, assuring the pathogen stable colonization.

The switch from acute to chronic infection mode, with all the related adaptive responses, is the results of a complex and finely regulated expression control. A main role seems to be played by the Gac/Rsm system, acting in response to environmental stimuli detected through the RetS sensor kinase and regulating several operons, including those involved in T3SS and T6SS secretion and biofilm formation (Goodman et al., 2004). Furthermore, *P. aeruginosa* tends to accumulate mutations over the infection development, and some genes have been described as mutation hotspots, revealing a convergent adaptation mechanism in the lung environment. Among the various target genes, the regulatory *mucA* and *retS* genes are the main mutated ones (Marvig et al., 2015).

A particular feature is that within one single patient different *P. aeruginosa* subpopulations and even different strains can coexist. Differences like colony morphology, motility, quorum sensing, protease activity, auxotrophy, siderophore levels, antibiotic resistance and growth profiles have been evidenced, even within the same morphotypes, in various strains, isolated in subsequent samples from the same patients and all belonging to the same clonal lineage (Workentine et al., 2013).

Moreover, the coexistence of different hyper-mutable lineages and sub-lineages within the same patients has been described, with a particular focus on the mutations that can confer a fitness advantage in the persistence in the lung environment (Feliziani et al., 2014).

3.3.4 Recurrent and chronic infections

Recurrency is the main characteristic of CF *P. aeruginosa* infections, presenting continuous pulmonary exacerbation episodes and the isolation of the pathogen even after long antibiotic treatment periods. The definitions of intermittent and chronic *P. aeruginosa* infections have been long debated and subjected to different revisions. The two main versions of infection categories correspond to the Copenaghen and the Leeds criteria (Pressler et al., 2011). According to the formers, an infection is defined intermittent when showing in a 6 months period at least one *P. aeruginosa* positive culture combined with normal levels of *P. aeruginosa* precipitated antibodies, whereas the chronic infection exhibits a persistent *P. aeruginosa* presence combined with two or more *P. aeruginosa* precipitating antibodies. According to the Leeds criteria, an intermittent infection reports less than 50% of cultured samples positive for *P. aeruginosa* over 1 year, while a chronic infection is characterized by more than 50% of samples positive for the pathogen presence. A patient is then considered free of *P. aeruginosa* infection if not showing a culture positive sample in the previous 12 months.

Finally, another version is constituted by the European consensus definition of chronic infection, characterized by three positive cultures over more than 6 months, with at least a 1-month interval between the samples (Proesmans et al., 2006).

The intermittent infection is an intermediate stage, when *P. aeruginosa* is alternatively isolated from sputum samples after antibiotic treatment. The main problem connected with this status is the question whether the patient is colonized by the pathogen de novo or if the antibiotic treatment fails in eradicating it and simply reduces its amount under the cultural assays limit of detection. The possibility of a bacterial persistence, undetected by routine cultural assays had been proposed by Schelstraete et al., (2010); later, Yum et

al., (2014) demonstrated that *P. aeruginosa* infection recurrency is mainly due to the relapse of prior morbid episodes, by detecting the same bacterial pulsotype in monitoring CF patients during a 4 years study and suggesting the pathogen presence even when not revealed by cultural identification.

An alternative hypothesis is that *P. aeruginosa* may be harbored in other airways niches, acting as an infection reservoir. Indeed, a crescent number of studies is focusing on the pathogen colonization of the sinus (Linnane et al., 2015), considered a favoring niche for bacterial proliferation, and the presence of viable and latent cells in pulmonary nodules ha salso been reported (Ronkainen et al., 2014).

The chronic infection rapidly and commonly develops in CF airways and is characterized by a wide antibiotic resistance and bacterial persistence in the lung environment. This infection stage selects for highly adapted *P. aeruginosa* populations that are almost unaffected by the antibiotic treatment and can promote pulmonary exacerbations. *P. aeruginosa* SCVs have been described as the main responsible for the infection relapse, due to their enhanced biofilm production; a new interest is being dedicated to their characterization and to the research of the genetic pathways leading to their formation, evaluating in particular the role of the high c-di-GMP levels present in *P. aeruginosa* biofilms (Evans, 2015). A further hypothesis (Amiri et al., 2015) is the development of a Viable But Non-Culturable (VBNC) population, undetected by routine cultural techniques, able to tolerate the antibiotic treatment and to cause the infection relapse. Chronically infected patients suffer from respiratory failure, especially when colonized by transmissible hypervirulent strains and must often be subjected to lung transplantation to survive (Srour et al., 2015).

CHAPTER I-MOLECULAR DETECTION OF DORMANT *P. aeruginosa* CELL FORMS IN CYSTIC FIBROSIS SPUTUM SAMPLES

1. INTRODUCTION

1.1 *Pseudomonas aeruginosa* infections clinical diagnosis

Pseudomonas aeruginosa pulmonary infection is the main cause of morbidity and mortality among CF patients (López-Causapé et al., 2015). The fast chronic development of the infective process, correlated with biofilm formation, is often characterized by the insurgence of atypical phenotypes, like SCVs, auxotrophic mutants (Anuj et al., 2009) and even slow growing non-culturable forms (Deschaght et al., 2013). These latters, in particular, hamper the effectiveness of the infection diagnosis, still performed by the cultural assays that are considered the gold standard for the routine diagnostics. The cultural approach is, indeed, cheap and easy to perform and can be supported by biochemical and mass spectometry identification. Moreover, a first study (Deschaght et al., 2009), comparing the routine diagnostic assays, performed by plating CF samples aliquots on different agar media (i.e. MC agar, cetrimide agar and blood agar), and culture-independent techniques did not show any significative difference in efficiency between these methods, supporting the use of the formers in the microbiology labotarories.

However, this approach carries a high risk of misidentification (Wellinghausen et al., 2005), and it usually takes more than 3 days to provide a reliable result (Xiaomeng et al., 2014). It was even reported that, in children affected by CF, the pulmonary infection occurred between 6 and 12 months before the pathogen was first isolated from respiratory secretions (West et al., 2002). Moreover, the development of different and unsual morphotypes has determined the partial withdrawal of culture-dependent methods (Douraghi et al., 2014). The cultural assays seem thus unsuitable to reliably represent the bacterial colonization in the CF lung.

The typical marks of the intermittent infections, with alternative Colture Positive (CP) and Colture Negative (CN) samples, and the relatedness of subsequent *P. aeruginosa* isolates (Yum et al., 2014) are the clear evidences of this concern.
The role of bacterial dormant forms in antibiotic treatment failure and in the infection relapse was already suggested (Høiby et al., 2010 B) and the general problem of unculturable bacteria detection has been recognised as deeply affecting the infection clinical management (Bittar and Rolain, 2010). Furthermore, Deschaght et al. (2013) evidenced their presence in the sputum samples of monitored CF patients, independently from the clinical conditions: indeed, good clinical respiratory parameters were correlated with a reduced number of both culturable and non-culturable *P. aeruginosa* cells, however a persister population, undetected by cultural assays, was always present. The main threat represented by these forms is the possibility of their reactivation and/or conversion to the vegetative form, able to cause pulmonary exacerbation. Thus, the need for a culture-independent, efficient, reproducible and reliable diagnostic protocol is pivotal to counteract *P. aeruginosa* infection.

1.2 The molecular techniques and their impact on the pathogen detection

The development of molecular techniques in the latest years has revolutionized the whole biology, assuring a deeper and more accurate understanding of most genetical and biochemical pathways. In microbiology, it did not only elucidate the genetic bases of microorganisms lifestyle and pathogenicity, but allowed even the detection and identification of new bacterial species, previously unknown or confused with closely-related species. Nowadays, the molecular detection is applied in pathogen identification in food (Hyeon and Deng, 2016) and in the environment (Volpe et al., 2016) and has proved to be essential in the determination of microbiomes (Caverly et al., 2015).

In the specific case of CF, it is true that some laboratories perform daily Real-Time PCR assays rather than biochemical tests for pathogen identification (Burns and Rolain, 2014), but the cultural assays still remain the gold standard.
Indeed, various protocols have been proposed to correctly identify *P. aeruginosa* recovered from CF specimens (Qin et al., 2003; Lavenir et al., 2007; Motoshima et al., 2007; Le Gall et al., 2013), targeting different conserved genes; however none of these protocols has been considered satisfactory. Infact, only a few articles dealt with the problem of the pathogen direct detection (Fothergill et al., 2013; Le Gall et al., 2013) and many protocols are affected by the lack of discrimination between live and dead cells (Kaushik and Balasubramanian, 2013).
Some authors have overcome this issue by adopting a sample pre-treatment with Propidium MonoAzide (PMA) before processing it for DNA extraction (Nocker and Camper, 2008; Rogers et al., 2013; Tavernier and Coenye, 2015), but a study performed on *Legionella spp,* (Taylor et al., 2014) reported several limitations of this method when dealing with samples presenting biofilms. Thus, microbiologists and clinicians are still looking for an alternative culture-independent method able to perform an early and reliable direct detection of *P. aeruginosa* in CF samples.

1.3 Aims of the study

The aim of this study was to develop a culture-independent approach to reliably detect and quantify *P. aeruginosa* viable culturable and non-culturable cells in CF sputum samples. In a previous study (Amiri et al., 2015) the pathogen detection in CP and most of all CN samples was performed using Real-Time PCR assays targeting specific and already reported genes, i.e. *ecfX*, *gyrB* and *oprL*. The obtained results indicated the first target as the most sensitive and reliable. The *ecfX* gene codes for a species-specific σ-factor, present as 19 copies in *P. aeruginosa* genome (Lavenir et al., 2007), responsible for the expression of various proteins with an extra-cytoplasmic localization and probably involved in haem-uptake and virulence factors production. The combined DNA extraction/*ecfX*-targeting qPCR protocol has been improved by adding a DNase I pre-treatment of the sample before DNA extraction; DNase had already been described in dealing with *P. aeruginosa* CF lung infections as a key factor to functionalize antibiotic nanoparticles (Baelo et al., 2015; Deacon et al., 2015) or to dissolve sputum solids (Gustave et al., 2013).

In this particular approach, it was used in order to avoid eDNA recovery and its interference in *P. aeruginosa* quantification and it was tested to validate the protocol efficiency and reliability. A particular focus was dedicated in identifying unculturable *P. aeruginosa* cell forms, to investigate, in subsequent studies, their involvement in the infection relapse.

2. MATERIAL AND METHODS

2.1 Strains and samples collection

Fifty random sputum samples, either resulted CP or CN for *P. aeruginosa* by routine diagnostic cultural assays, were taken among those coming from the CF centre of Marche region, Italy, from March 2015 to May 2016. Samples were collected in sterile cups and were maintained at 4°C until analysis. One-hundred fifteen additional *P. aeruginosa* isolates (51 CF clinical isolates, 55 non-CF clinical isolates and 9 environmental ones) plus 7 Gram-negative non *P. aeruginosa* isolates were collected from Microbiology laboratoriess of AO "Ospedali Riuniti di Ancona" (Italy), AO "Ospedale A. Murri" (Fermo, Italy) and from "Arpam" (Pesaro, Italy). Gram-negative isolates comprehended typical CF pathogens (i.e. *Achromobacter xylodoxidans, Burkholderia cepacia* and *Stenotrophomonas maltophila*) and other similar species. These bacterial isolates were identified as follows:

- 51 CF clinical *P. aeruginosa* isolates by mass spectrometry (MALDI TOF, VITEK-MS, BioMèrieux)
- 55 non CF *P. aeruginosa* isolates and Gram-negative isolates by biochemical identification (WalkAway system, MiscroScan Walk Away 40, Beckman Coulter, California, USA)
- 9 environmental *P. aeruginosa* isolates by biochemical identification through API 20NE strips (BioMérieux)

Further 3 Gram-negative non *P. aeruginosa* isolates (i.e. *Klebsiella pneumonia*e, *Proteus mirabilis* and *Escherichia coli*), included in this study, belong to the Microbiology laboratory strain collection of the Department of Life and Environmental Sciences (DiSVA) of Polytechnic university of Marche (UNIVPM).

All strains were cultured on Mueller-Hinton (MH) agar plates, subcultured in MC agar and conserved in MH broth supplemented with glycerol 20% at -80°C.

2.2 Sputum samples cultural assays

Sputasol (Oxoid, Hampshire, UK) was added in 1:1 ratio to samples before being processed. Routine cultural assays were performed following the guidelines described by Gilligan (2007), and reported by the UK Consensus Document (2010), using a combination of MC agar, chocolate agar and Columbia blood agar. The final identification was based on mass spectrometry, as described above. The *P. aeruginosa* abundance was determined as falling into the following ranges (CFU/ml): $<10^2$ (i.e. CN), 10^2-10^3; 10^3-10^4; 10^4-10^5; 10^5-10^6.

2.3 Spiking experiments

Spiking experiments were performed as described by Amiri et al. (2015). Briefly, 1 ml aliquots of a sputum sample, previously tested negative for *P. aeruginosa* presence by both cultural and molecular methods, were spiked with *P. aeruginosa* ATCC 9027 cultures containing $3x10^6$ cells/ml. Inocula comprehended log phase and live/dead cultures; for the latters, a proportion of 1:10 between live and dead, heat-treated (i.e. incubation at 75°C, 15 min) cells was used. Total DNA was extracted from each spiked sample and examined in *P. aeruginosa* qPCR assays.

2.4 DNA manipulation

2.4.1 DNase I digestion

To remove eDNA and to avoid related false positive results, samples were digested with DNase I before the extraction process. One ml aliquots were centrifuged at 15000xg, at 4°C, for 15 min, resuspended in 500 µl of phosphate buffered saline (PBS) and treated with 18 U of DNase I (Ambion, California, USA) at 37°C for 30 min. The enzyme was then inactivated by incubation at 75°C for 10 min and the sample washed in 1ml of PBS and processed for DNA extraction. Tested samples included a live/dead (1:10) *P. aeruginosa* ATCC 9027 broth culture, spiked sputum samples, prepeared as described above, and 27/50 CF sputum samples. All samples were analysed in duplicate, comparing the molecular results obtained with a DNase-treated aliquot and an untreated one.

2.4.2 DNA extraction

Total DNA was extracted from each sample using the QiAmp DNA Mini Kit (Qiagen, Hilden, Germany), as described by Amiri et al. (2015), following the manufacturer instructions and eluted in a final elution volume of 80 µl. The amount and purity of extracted DNA were verified with a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, NC, USA) and by agarose gel electrophoresis.

Total DNA from bacterial isolates and *P. aeruginosa* ATCC 9027 broth cultures was extracted preparing a crude cell lysate, as previously described (Hynes et al., 1992).

2.5 Real-Time PCR assays

2.5.1 The *ecfX* target gene

The *ecfX*-targeting protocol developed by Amiri et al., (2015) was selected for further improvements, since reporting a greater sensitivity (i.e. 60 cells/ml) and a major number of CN PCR-positive samples (i.e. 6/33 sputum samples). The amplified region (i.e. 145 bp) used to detect *P. aeruginosa* cells was obtained by testing a new combination of primer pairs that had been previously reported, specifically ecfX-F 5'-AGCGTTCGTCCTGCACAAGT-3' (Clifford and al., 2012) and ecfX-R 5'-TCATCCTTCGCCTCCCTG-3' (Lavenir et al., 2007). Briefly, reactions were carried out in a total volume of 20 µl containing 0.2 µM of each primer, 10µl of 2×Rotor-Gene SYBR Green PCR master mix (Qiagen) and 2µl DNA. Cycling conditions were 95 °C for 5 min, followed by 33 cycles of 95 °C for 10s, 61°C (i.e. annealing temperature) for 30s and 72°C for 20s. Melting analysis was run by ramping the temperature from 59°C to 95°C (0.5°C/10s) and using Qiagen's Rotor-Gene Q MDx software. Each reaction was run in triplicate. DNA of *P. aeruginosa* ATCC 9027 and RNase-Free water were used as positive and negative control respectively. The reaction efficiency was calculated by using Qiagen's Rotor-Gene Q MDx software.

2.5.2 Sensitivity assays

The sensitivity of the *ecfX*-targeting protocol had already been determined by Amiri et al. (2015); furthermore, a calibration curve was built (Amiri et al., unpublished data), in order to correctly quantify *P. aeruginosa* abundance, using scalar dilutions of an *ecfX* amplicon obtained from DNA of a broth culture of *P. aeruginosa* ATCC 9027. The amplicon was purified by Gene Elute PCR Clean-up kit (Sigma-Aldrich, Saint Louis, MO, USA) and quantified by ND 1000 Nanodrop; dilutions containing from

10^{-6} to 10^{-9} ng/reaction were used as standards. The protocol Limit Of Detection (LOD) was determined as described by Bustin et al., (2009). Each reaction was run in triplicate.

2.5.3 Specificity assays

To confirm the *ecfX* amplicon conservation and assess its reliability as unique specie-specific target gene, 115 *P. aeruginosa* isolates and the 10 Gram-negative isolates were tested by classical PCR, using the same primer pair described for the Real-Time PCR assays and the Dream-Taq Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The amplification conditions comprehended 10 min at 95°C, followed by 35 cicles of 30s at 94°C (denaturation), 30s at 61°C (annealing), 45s at 72°C (elongation) and a final elongation step of 5 min at 72°C. *P. aeruginosa* ATCC 9027 DNA was used as positive control. All amplification products were checked by gel electrophoresis and the percentage of PCR-positive *P. aeruginosa* strains was calculated. The presence of crossreaction with the other bacterial species was assessed as well.

2.5.4 Cystic fibrosis sputum samples analysis

The spiked sputum samples and the 50 CF clinical ones were all analysed for *P. aeruginosa* presence and abundance. The latter was calculated as previously described (Di Cesare et al., 2013): briefly, it was estimated that each *P. aeruginosa* cell contains 3×10^{-9} ng of the *ecfX* target sequence, a value obtained multiplying the weight of 1 DNA bp (i.e 1.095×10^{-12} ng) for the length of the amplicon (i.e. 145 bp) and for the number of *ecfX* copies contained in the *P. aeruginosa* genome (i.e. 19). The amount of cells/ml contained in each sample was calculated by dividing the weight of the amplicon by this value and considering the initial 1:2 dilution with Sputasol. The obtained bacterial amount was then compared with the initial inoculum for the spiked samples and with the

culture counts for the clinical ones. In the first case, the molecular count proved the reliability of the combined DNA extraction/qPCR protocol in quantifying *P. aeruginosa* abundance; in the latter, it was meant to assess the presence of a VBNC subpopulation, not revealed by the routine cultural exams. The two counts, i.e. cultural and molecular ones, were considered comparable when the discrepancy was $<0{,}5\log_{10}$.

3. RESULTS AND DISCUSSION

3.1 Sputum samples cultural analysis

Among the 50 collected CF sputum samples, 17 resulted CN (CF1-3, CF12-19, CF31, CF33, CF42, CF48-50) and 33 CP, reporting the following cultural counts:

- 10^2-10^3 for 4 samples (CF9, CF28, CF32 and CF35)
- 10^3-10^4 for 3 samples (CF4, CF29 and CF37)
- 10^4-10^5 for 8 samples (CF5-6, CF20-21, CF23-24, CF40 and CF43)
- 10^5-10^6 for 18 samples (CF8, CF10-11, CF22, CF25-27, CF30, CF34, CF36, CF38-39, CF41, CF44-47)

3.2 Evaluation of DNase I treatment and DNA extraction efficiency

The DNase I pre-treatment of sputum samples was first evaluated on reference samples, i.e. a live/dead *P. aeruginosa* ATCC 9027 broth culture containing $3x10^6$ cells/ml and sputum samples spiked with the same amount of a log phase and a live/dead *P. aeruginosa* ATCC 9027 broth cultures. Results are reported in Fig. 6.

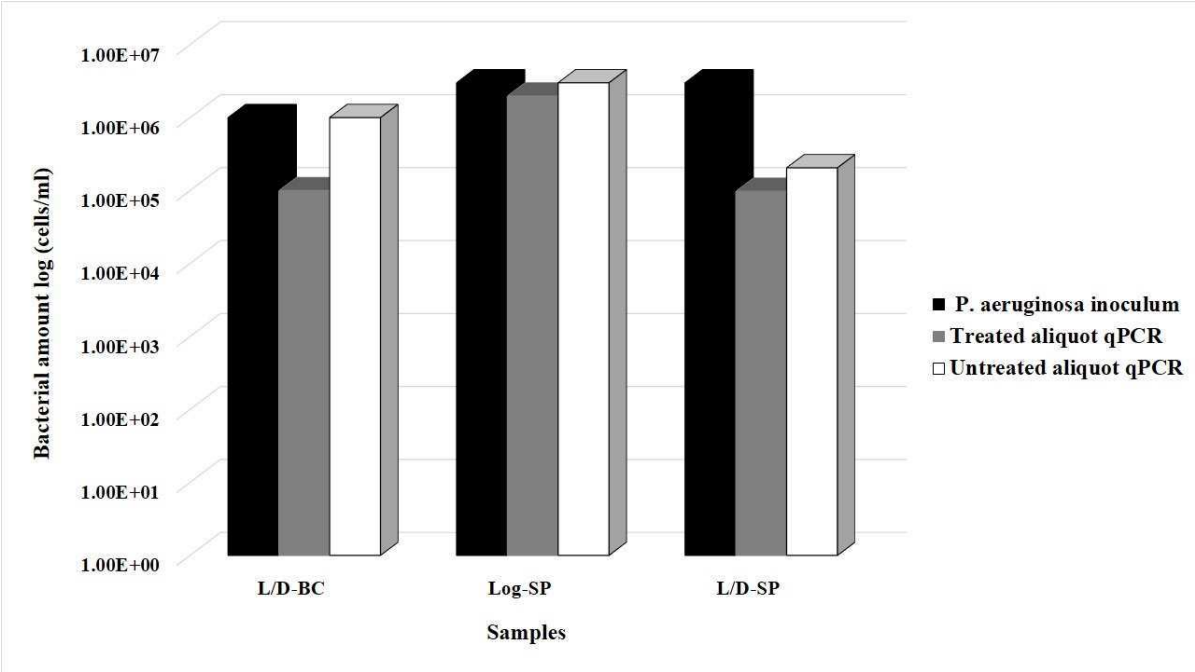

Fig. 6. Effect of DNase I treatment on *P. aeruginosa* quantification. The DNase I treatment efficiency was first evaluated on a live/dead (1:10) *P. aeruginosa* ATCC 9027 broth culture (L/D-BC) and 2 spiked samples, one with a log phase (Log-SP) and one with a live/dead broth culture (L/D-SP).

As shown in the graph, only the *P. aeruginosa* broth culture evidenced a clear difference in the molecular *P. aeruginosa* quantification between the DNase treated and untreated aliquots. The effect of the DNase treatment was assured by total DNA recovery quantification, that exhibited always a lower amount in the treated aliquots, indicating the enzyme activity on eDNA (data not shown). It must be noticed, though, that the broth culture DNA was extracted as a crude cell lysate, not subjected to any kind of selection or filtration. The spiked samples were extracted using the Qiagen kit, based on the binding of the nucleic acid to the specific silica columns and on its retaining during the extraction process. An interesting result was provided by these samples, since the *P. aeruginosa* qPCR resulted comparable only to the live portion of the initial inoculum. Specifically, in the case of spiking with a log phase culture, the qPCR count perfectly matched with the whole inoculum, indicating that DNase I does not interact with live integer cells; whereas when the sputum was spiked with a live/dead culture, both the treated and untreated aliquots gave a qPCR result

matching with only the live portion of *P. aeruginosa* (i.e 1/10 of the whole inoculum). This is quite remarkable, since even the untreated aliquot did not detected the DNA related to the dead *P. aeruginosa* cells. Most likely, the *P. aeruginosa* eDNA present in the sputum sample is too small and damaged to be retained by the DNA extraction kit, so there is not interference in *P. aeruginosa* quantification and the qPCR results can be considered reliable. The subsequent analysis of 27/50 CF sputum samples, examined both as DNase treated and untreated, confirmed the results obtained with the reference samples, since not relevant differences between counts of the two kinds of aliquots was detected.

The reference samples also documented the QiAmp DNA Mini Kit efficiency in the extraction of bacterial DNA from the sputum samples, since the molecular quantification of the pathogen cell amount matched with the used inoculum.

3.3 Real-Time PCR sensitivity and specificity evaluation

The *ecfX*-targeting protocol reported good and reproducible results. The regression coefficients were close to one (R2 values: 0.998) and mean qPCR efficiency was 0.97±0.02. The calculated LOD was 5.2×10^{-9} ng/reaction, corresponding to 1.7 cells/reaction and indicating that the minimum quantifiable amount of *P. aeruginosa* by qPCR in the original sputum samples was 80 cells/ml, a value very close to the protocol sensitivity threshold reported by Amiri et al. (2015), i.e. 60 cells/ml.

The characterization of the 115 *P. aeruginosa* strains confirmed the *ecfX* target sequence conservation (Table 1): only 4 isolates, 3 isolated from CF sputum samples and 1 isolated from a clinical non CF sputum sample, resulted negative to *ecfX* -PCR, whereas the 96.6 % of isolates gave a positive result. No cross-reaction was noticed when testing other Gram-negative isolates for the same target gene, even in *P. aeruginosa*-related species such as *Stenotrophomonas maltophila* or *Burkholderia cepacia*.

Table 1. PCR analysis of the 115 *P. aeruginosa* and the other Gram-negative isolates

ISOLATE	*ecfX* PCR+	*ecfX* PCR-
Cystic Fibrosis *P. aeruginosa* clinical isolates (51)	48	3
P. aeruginosa clinical isolates (55)	54	1
P. aeruginosa environmental isolates (9)	9	0
Serratia marcescens (2)	0	2
Stenotrophomonas maltophila (2)	0	2
Burkholderia cepacia	0	1
Achromobacter xylosoxydans	0	1
Klebsiella pneumoniae	0	1
Morganella morganii	0	1
Proteus mirabilis	0	1
Escherichia coli	0	1

EcfX-PCR results of the analysed *P. aeruginosa* and Gram-negative isolates.

The *ecfX* gene had been already reported as species-specific for *P. aeruginosa* and had been used by other authors in identifying this microorganism (Colinon et al., 2013). However, since *P. aeruginosa* shows a high plastic genome, very susceptible to mutations, the use of two or three target genes, in order to avoid false negative results, is often recommended; among others, the *gyrB* and *oprL* gene are the most commonly reported as reliable in the pathogen detection (Anuj et al., 2009). In the study performed by Amiri et al. (2015), the *oprL*-targeting protocol did not show the required specificity for a reliable diagnosis, whereas the *gyrB* gene reported a too high threshold value (i.e. 10^2 cells/ml), comparable and not more sensitive than cultural assays cut-off. These conditions are not suitable to detect unculturable *P. aeruginosa* forms, especially when present in a low amount. The *ecfX* gene reported a greater sensitivity and the target sequence seems well conserved among different strains, even recovered from different sources, and not shared by other similar bacterial species, even those traditionally considered *P. aeruginosa*-related (Coenye et al., 2001; Falagas et

al., 2008). Although further efforts are being dedicated to the improvement of both sensitivity and specificity of this protocol, the obtained results seem already valuable to reliably implement the routinely performed *P. aeruginosa* infection diagnosis.

3.4 Cystic fibrosis sputum samples analysis

The molecular analysis of the 50 CF sputum samples evidenced the efficiency of the developed *ecfX*-protocol, as shown in Tab. 2.

Table 2. Molecular characterization of the 50 analyzed CF sputum samples

SAMPLES (50)	qPCR+	qPCR-	qPCR>PC
CP (33)	33/33	0/33	10/33
CN (17)	2/17	15/17	/

Molecular analysis of the 50 CF sputum samples. Number of Colture Negative (CN) and Colture Positive (CP) samples resulted ecfX-qPCR positive (qPCR+) or negative (qPCR-); the number of CP samples showing qPCR counts higher than plate counts (qPCR>PC) is also reported.

All the CP samples reported a positive *ecfX*-qPCR outcome, excluding false negative results and confirming the target gene suitability in detecting *P. aeruginosa* presence. However, 10 of these samples, together with 2 CN ones, reported interesting data, showing, in the first case, higher qPCR counts than cultural quantification and, in the latter, a positive PCR result with a negative cultural one. In both cases the presence of a viable *P. aeruginosa* subpopulation, undetected by routine cultural assays, was evidenced. Indeed, the interference in the pathogen quantification of eDNA, known to be a structural element of *P. aeruginosa* biofilms (Novotny et al., 2016), can be excluded, considering the results obtained with the DNA extraction based on the Qiagen kit, and the differences observed in the two methods of bacterial counting can be attributed to the presence of persister non-culturable forms. After all, their presence had already been reported during the monitoring of CF patients (Deschaght et al.,

2013), and their possible role in the infection relapse had been suggested (Yum et al., 2014). The factors responsible for their induction are still debated, but it is well known that ferrous iron plays a significant role in most *P. aeruginosa* metabolic pathways, including biofilm development (Barton et al., 1996), and had been reported as significant component of the metal bioavailability in CF airways (Hunter et al., 2013). Moreover, a previous study performed on *S. aureus* (Pasquaroli et al., 2013) demonstrated that antibiotic treatment could be responsible for the dormant state induction; since CF patients are always subjected to continuous and aggressive antibiotic treatment, especially when affected by chronic *P. aeruginosa* infections, it is reasonable to think of this non-culturable state as a bacterial response to antibiotic pressure, representing a stress condition.

In the case of the 2 CN samples, both exhibiting low *P. aeruginosa* amounts, an alternative explanation could be the selection of a persister subpopulation, antibiotic tolerant (Lewis, 2010) and present at a too low concentration to be detected by the cultural assays.

The relationship between *P. aeruginosa* biofilms and antibiotics was subsequently evaluated, with a particular focus on the role of subinhibitory antibiotic concentrations.

4. CONCLUDING REMARKS

P. aeruginosa infection diagnosis represents one of the main concerns in CF pathology, and especially the demand for alternative techniques able to provide an early, efficient and reliable result is assuming a growing interest in the prevention of *P. aeruginosa* chronic infections. The developed protocol, based on the combination of an efficient DNA extraction process and a sensitive and species-specific qPCR, perfectly fits in this topic, representing a useful implement to the already used cultural techniques. Its standardization could lead to an earlier detection of the pathogen, an effective infection treatment and, hopefully, to its eradication. Furthermore, it has exposed the presence of *P. aeruginosa* non-culturable forms in CF sputum samples, probably involved in the infection relapse and responsible for the failed pathogen eradication. This theme represents a new achievement in the understanding of *P. aeruginosa* lung colonization and population dynamics, arising the search for the factors involved in their control.

CHAPTER II-INDUCTION OF *P. aeruginosa* DORMANT FORMS IN AN *in vitro* BIOFILM MODEL

1. INTRODUCTION

1.1 *Pseudomonas aeruginosa* biofilms and VBNC forms

Biofilm lifestyle represents a winning strategy for bacteria in developing an infection, providing both an ideal environment for proliferation and protection against the host immune defenses. A particular advantage, especially in *P. aeruginosa*, consists in the differentiation of bacterial subpopulations able to adapat to different adverse conditions (Chiang et al., 2012). The main reported case is the distinction of a metabolic active population, able to divide and to continue the infection development, and a slow-growing one, typical of the deeper layers of biofilm. Both populations show an increased antibiotic tolerance, despite through different mechanisms. The former relies on motility and quorum sensing communication to evade the action of antibiotics like colistin (Chua et al., 2016), as well as on aspecific resistance mechanisms potentiation, such as efflux pumps (Pamp et al., 2008). The latter, also known as persister cells, resists and tolerates the antibiotic action thanks to its low metabolism, allowing the biofilm ripopulation once the antibiotic pressure is over (Lewis, 2010). Among persisters, a particul mention has to be dedicated to the VBNC state.

VBNC cell forms are defined as unable to grow and to form colonies on agar media (Oliver, 2005) and are distinguished from other persisters and atypical phenotypes for this reason. They present a very low and basic metabolism, as the normal persister cells, and their antibiotic tolerance is not based on specific mutations or acquisition of new resistance determinants. Their presence can be evidenced through vitality assays, performed by specific cell staining methods (i.e. live/dead or DAPI staining) or by enzymatic assays, searching for the activity of specific intracellular proteins. In the latest years, the use of the molecular methods has allowed not only their detection (Deschaght et al., 2013),

but even the characterization of their specific genetic expression (Pasquaroli et al., 2013).

Some specific features of these forms have been described (Oliver, 2005): VBNC forms exhibit dwarfing and substantial metabolic changes, like reduction of nutrient transport, respiration rates and macromolecular synthesis. However, they still retain a basic synthesis activity and, surprisingly, they present high ATP levels. Other typical changes seem to be the modification of the cytoplasmic membrane fatty acids composition and the increase of cross-linkings in the cell wall.

A singular feature, that has been well characterized in some *Pseudomonas* species, is the cell plasmid carriage, that seems to play a crucial role in the induction, and most of all, in the maintenance of the VBNC state (McDougald et al., 1995).

The particular interest dedicated to these bacterial forms is due to their supposed role in recurrent and chronic infections. Infact, if the persister cells have been demonstrated to be a bacterial survival assurance typical of chronic infections, the role of the VBNC forms is still debated. Since they present a near dormancy metabolic state, it is unprobable that they can express virulence factors and/or cause infections; however, VBNC forms are known for their possibility to "resuscitate", i.e. convert again to the vegetative form, fully able to develop infective processes. This capability is still under assessment, and another definition of the VBNC state is, indeed, a pre-mortem one, leading to bacterial dead. It has actually been proposed that persister cells and VBNC forms belong to the same response to environmental stress, representing different stages of the same phenomenon (Ayrapetyan et al., 2015), as shown in Fig. 7.

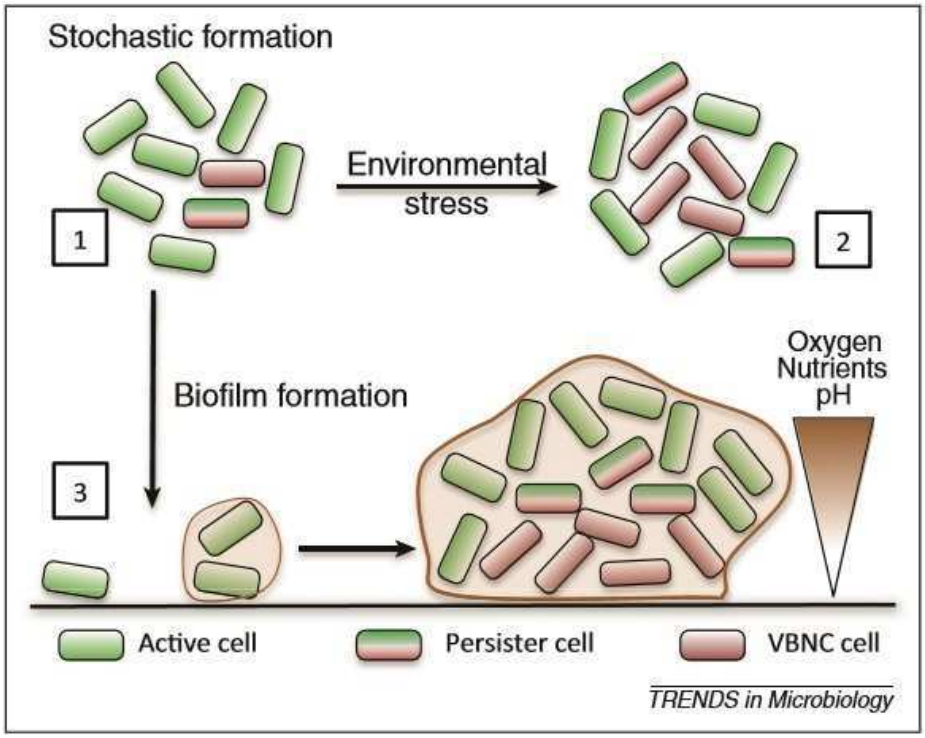

Fig. 7. Mechanisms of persister and VBNC cells formation. The induction of dormant cell forms is attributed to 3 different mechanisms, mainly stochastic formation (1), response to environmental stress (2) or differentiation of specific subpopulations within biofilms (3).

However, it is still not clear which exact factors can induce the VBNC forms and, subsequently, which ones can riactivate them. Some inducers and genetic pathways have been proposed, but the finest details of this state development have still to be elucidated.

1.2 VBNC forms inducers

The identification of the main VBNC inducers has raised a growing interest in elucidating the genetic pathways responsible for this phenomenon.

Some factors involved in the VBNC forms development are natural stimuli (Oliver, 2005), underlining that this state is a general stress response mechanism, that has found a particular meaning in the nosocomial environment. Among these, the main ones are:

- Starvation
- Atypical temperatures (i.e. out of the range of growth)
- High osmotic concentrations
- Exposure to white light

Regarding starvation, a genetic pathway, related to the post-transcriptional control of the Crc protein, has been proposed (Zhang et al., 2012) in *P. aeruginosa*: according to this model, the protein responds to nutrient depletion and catabolite repression, mediating the differentiation between an active and a slow-metabolism subpopulations. Further studies have evidenced the connection between this pathway and the PQS system, revealing a complex regulatory system involved in virulence, biofilm formation and, subsequently, specific phenotypes development (Zhang et al., 2013).

Another particular condition, connected with the biofilm lifestyle, is the hypoxia (Williamson et al., 2012), experienced by the cells present at the bottom of biofilms; this stress stimulus seems to be responsible for the expression of ribosome hibernation factors, in particular encoded by the *rmf* gene, that maintain active the ribosomes and assure the survival of the VBNC forms even for a long period.

Finally, it has been reported that VBNC forms can be induced even during bactericidal processes. Specifically, milk pasteurization (Gunasekera et al.,

2002) and UV disinfection (Zhang et al., 2015) have been described as inducers of *Pseudomonas* VBNC forms.

An interesting observation was provided by Pasquaroli et al. (2013), describing how antibiotic treatment itself constitutes a stress condition able to induce the VBNC state. This theory, combined with the hormesis one (Davies et al., 2006), could perfectly explain the implication of VBNC forms in CF pulmonary infection recurrency.

1.3 The hormesis and the subhinibitory antibiotics concentrations

The concept of hormesis has been defined as biological response to environmental signals or stresses, characterized by biphasic dose-response relationships, i.e. low dose-stimulation and high-dose inhibition (Davies et al., 2006). The phenomenon is well represented in Fig. 8.

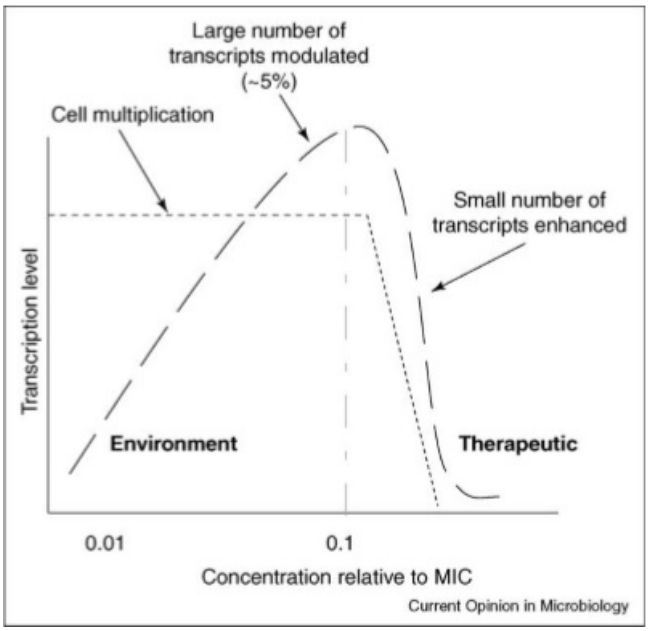

Fig 8. The hormetic antibiotic effect. The growth inhibition, represented by the decreased cell multiplication, and the trascription levels are related to the antibiotic concentrations, showing a stimulatory effect at sub-MIC doses and an inhibitory one at higher doses. Authors suggest that the formers are typical environmental concentrations, whereas the latters find more applications in the nosocomial environment.

The stimulatory effect is achieved through expression modulation; there is no conserved pattern, as bacterial response can vary according to the antibiotic used, to its mechanism of action and, among the range of sublethal concentrations, to its dose.

Typical examples, specific for *P. aeruginosa* hormetic phenomena, comprehend:

- Induction of prophage genes after exposure to fluoroquinolones (Brazas and Hancock, 2005)
- Motility repression, induction of β-lactamases, alginate and peptidoglycan synthesis after exposure to imipenem (Bagge et al., 2004)
- Increase of biofilm formation in presence of aminoglycosides (Hoffman et al., 2005)
- Repression of virulence factors expression mediated by macrolides (Nalca et al., 2006)
- Decreased biofilm production and motility due to mupirocin presence (Horii et al., 2003; Ishikawa and Horii, 2005)

The ribosome seems to constitute the perfect machinery for the hormesis phenomenon; indeed various antimicrobials target it and various expression profiles have been associated with antibiotic-specific inhibition mechanisms; moreover, it has been supposed that ribosomes can mediate the hormetic effects thanks to the action of second messengers and other signal molecules (Davies et al., 2006). Among them, ppGpp (Guanosine tetraphosphate) seems to play a great role, connecting the activity of different cell regulator proteins, such RelA, SpoT, Crp and RpoS.

An interesting hypothesis is the involvement of hormesis in the differentiation of bacterial subpopulations within biofilms. It is, indeed, possible that sub-MIC antibiotic concentrations can mediate specific expression responses, causing the metabolism slow-down, typical of persister and even VBNC forms. Although there is a growing interest for these particular cell forms, especially for their

implication in chronic infections, few studies have investigated whether they constitute a hormetic adaptive response.

1.4 Aims of the study

The aim of this study was to develop an *in vitro P. aeruginosa* biofilm model, resembling the conditions of a CF chronic pulmonary infection. The mature biofilm was then subjected to different stress conditions, in order to verify the VBNC state induction, explaining the results previously obtained by the CF sputum samples analysis, and to identify the main inducers of this state.

Stress conditions included:

- Starvation, observed in the lower layers of mature biofilms, characterized by nutrient depletion
- Exposure to subinhibitory antibiotic concentrations, mimicking the CF patients lung conditions, continuously affected by antibiotic treatment

Ciprofloxacin and tobramycin were chosen as tested antibiotics, since reported as those commonly adopted in *P. aeruginosa* airways infections treatment and the most efficient ones (Stockmann et al., 2013; Stanojevic et al., 2014).

The obtained results gave new insights on the role of antibiotics in the induction of the VBNC state, revealing the possible connection between antibiotic treatment, bacterial persistence and infection recurrency.

2. MATERIAL AND METHODS

2.1 Bacterial strains, bacteriological media and antibiotics

The strain *P. aeruginosa* C24, isolated in 2015 from a chronic CF patient and resulted positive in the *ecfX* gene specificity assays, was used as study model for the development of *in vitro* biofilm models. This strain proved to be an XDR *P. aeruginosa* isolate, it was susceptible only to 1/14 (i.e. colistin) tested antibiotics in routine antibiotic susceptibility tests performed in the Microbiology laboratory of AO "Ospedali Riuniti di Ancona" (Italy).

The strain *P. aeruginosa* ATCC 27853 belongs to the strain collection of the Microbiology lab of the Department of Life and Environmental Sciences (DiSVA) of Polytechnic Univeristy of Marche (UNIVPM), Ancona, Italy.

Strains were cultured in MH agar, subcultured in MC or *Pseudomonas* agar base additioned with the *Pseudomonas* CFC selective supplement (Oxoid Ltd., Basingstoke, UK), and conserved in Brain Hearth infusion (BH) broth supplemented with glycerol 20% at -80°C.

Cystine-Lactose-Electrolyte Deficient (CLED) agar, adopted in cultural assays, and the antibiotics ciprofloxacin (fluoroquinolone) and tobramycin (aminoglycoside) were all purchased from Sigma-Aldrich (Saint Luis, Missouri, USA).

NonNutrient (NN) broth was a suspension of M9 salts 1x, $MgSO_4$ 2mM and $CaCl_2$ 0.1 mM in water.

2.2 *P. aeruginosa* C24 characterization

2.2.1 Biofilm production assay

P. aeruginosa C24 biofilm production was assessed by a biofilm plate assay, performed as described by Baldassarri et al. (1996). Briefly, *P. aeruginosa* C24 was grown overnight at 37°C in Triptone Soy Broth additioned with 1% glucose (TSBG), then 20 µl of this culture were transferred in a 96-wells-flat bottom microtiter plate and diluted 1:10 to a final volume of 200 µl with fresh TSBG. The plate was incubated for 18 h at 37°C, then washed three times with PBS (200 µl) and dried at 60°C for 1h. Bacterial biofilm was then stained with crystal violet 2% for 2-5 min, washed three times with water and dried again for 10 min at 60°C. Crystal violet was removed adding ethanol and the OD_{570} was measured by Spectrophotometer S-30, Boeco, Germany. The strain was tested in triplicate and water was used as blank. According to the obtained OD_{570} values, the strain was considered:

- Strong biofilm producer ($OD_{570} > 0.240$)
- Weak biofilm producer ($0.120 < OD_{570} < 0.240$)
- Non biofilm producer ($OD_{570} < 0.120$)

2.2.2 Minimal Inhibitory Concentration determination assays

Minimal Inhibitory Concentration (MIC) determination assays were performed with the microdilution method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). Briefly, bacterial strains grown overnight in MH broth at 37°C were diluted in cation adjusted MH broth (MHII) till reaching an OD_{625}=0.1, corresponding to around 10^7 CFU/ml in *P. aeruginosa*. Cultures were further diluted 1:100, then 50 µl aliquots were transferred in a 96-wells microtiter plate, containing MHII broth additioned with scalar 2-fold dilutions of the tested antibiotics (final volume 100 µl). Each line of the plate contained even

an antibiotic-free well, used as bacterial growth control. The plate was incubated for 18-24 h at 37°C. The lowest antibiotic concentration causing the absence of visible bacterial growth was considered the MIC. *P. aeruginosa* ATCC 27853 was used as reference strain. Both antibiotics were tested using concentrations from 0.125 to 128 µg/ml.

2.3 *In vitro* biofilm model development and dormant state induction

In vitro P. aeruginosa biofilms were developed in 24-wells microtiter plates, mimicking the conditions of a chronic *P. aeruginosa* pulmonary infection. *P. aeruginosa* C24 was grown overnight in TSBG at 37°C, then diluted 1:25 in fresh TSBG and let grow at 37°C with shaking for about 3 h, till reaching an OD_{650}=0.5-0.6. The colture was diluted again in fresh TSBG, adjusting the OD_{650} at a value of 0.1. One ml aliquots were then transferred in each well of a 24-wells microtiter plate and incubated at 37°C for 48 h. The culture medium was refreshed after the first 24 h with fresh TSBG.

The biofilm was subjected to different stress conditions:

- Starvation (growth in NN broth);
- Starvation combined with subinhibitory (1/4x MIC) antibiotic (ciprofloxacin or tobramycin) exposure.

The lack of nutrients has been well described in the deeper layers of *P. aeruginosa* biofilms (Sanchez et al., 2013), and a continuous antibiotic pressure is due to the aggressive antibiotic treatment applied to chronic patients (Máiz et al., 2013).

Biofilms were refreshed once a week, by removing the culture medium and adding fresh NN broth unsupplemented/supplemented with antibiotics.

2.4 Culturability and viability detection

Biofilms subjected to the various stress conditions were examined at different time points for a total period of 170 days to assess *P. aeruginosa* C24 viability and culturability. We considered as the starting point (T0) the time of the shift from nutrient (TSBG) to NN broth unsupplemented/supplemented with antibiotics. One-well biofilm for each condition was detached mechanically by vigoursly pipetting the medium in the well and then washing it with 1 ml of water; the whole suspension was transferred in a 15 ml falcon tube and, after centrifugation at 10000xg for 15 min, the pellet resuspended in 2 ml of PBS. Five-hundred µl aliquots of detached biofilms were used in the subsequent analyses.

2.4.1 Plate count method

Five hundred µl of detached biofilms were used for plate count assays. They were serially diluted (10-fold dilutions) and 100 µl of undiluted and diluted biofilms were plated on CLED agar plates. Plates were incubated at 37°C for 72h; CFU counts were performed at 24 and 72h, comparing the two counts and focusing on the presence of atypical phenotypes.

No recovery of bacterial colonies from the undiluted sample after 72h incubation at 37°C was stated as loss of culturability.

2.4.2 Epifluorescence microscopy assays

Epifluorescence microscopy assays were performed as described by Zandri et al. (2012), using 500 µl of detached biofilms. Briefly, biofilm aliquots were coloured by live/dead staining using SYBR Green I (Ivitrogen, Eugene, OR, USA, final concentration 1x) e propidium iodide (Sigma-Aldrich, Saint Louis Missouri, USA, final concentration 40µg/ml). The suspension was incubated at room temperature for 25 min in the dark and then filtered through black polycarbonate filters (0.22 µm, Millipore, Burlington, Massachusetts, USA), subsequently added on glass microscope slides. To prevent epifluorescence decay, 20 µl of antifade solution (50% di glycerol, 50% di PBS and 0.5% abscorbic acid) were added to both the top and the bottom of each filter. Glass microscope slides were finally observed at epifluoresce microscope Eclipse 50i (Nikon) at 100x magnification, with a grid objective and using specific filters; in particular, SYBR Green epifluorescence was observed at 450-490 nm (green fluorescence), whereas propidium iodide one at 510-590 nm (red fluorescence). Live and dead cells concentrations were determined dividing the number of counted cells (at least 400 cells) for the number of observed optical fields and multiplying the obtained value for the microscope convertion factor (i.e. 31360), resulted from multiplying the filter area (i.e. 490 mm^2) for the grid area (i.e. 0,015625mm^2). The final amount was further multiplied for 2, to obtain the cell concentration in 1 ml sample. Live and dead *P. aeruginosa* amounts were recorded starting from T60 and compared to the cultural counts.

2.4.3 qPCR quantification

Five-hundred µl of detached biofilm were processed for DNA extraction and *ecfX*-targeting qPCR quantification. The aliquots were centrifuged at 15000xg for 15 min, then resuspended in 100 µl of PBS and processed with the Qiamp DNA kit (Qiagen), as previously described (Chapter 1-Material and methods-DNA manipulation-DNA extraction). During the cell lysis step, the sample was subjected to 3 cycles of sonication for 1 min and incubation in ice for 1 min, to facilitate the lysis. This step was even prolonged from 2 to 24h, then the sample was processed following the manufacturer indications. The extracted DNA was quantified by *ecfX*-qPCR as previously described (Chapter 1-Material and methods-Real-Time PCR assays-The *ecfX* target gene). From T60, the *P. aeruginosa* molecular quantification was compared to the results obtained by culture and microscopy, in order to reveal the presence of a persistent non-culturable *P. aeruginosa* subpopulation.

3. RESULTS AND DISCUSSION

3.1 *P. aeruginosa* C24 characterization

In order to develop a suitable *in vitro* model, mimicking the *P. aeruginosa* pulmonary biofilm in CF chronic infection, at first we assessed the abilities of *P. aeruginosa* C24 to form biofilm and to resist to antibiotic action.

P. aeruginosa C24 resulted to be a strong (OD_{570}=0.76) biofilm producer and to be resistant to both tobramycin (MIC, 32 µg/ml) and ciprofloxacin (MIC, 4 µg/ml); all these features are typical of chronic infection isolates, thus confirming the suitability of the selected strain for the aim of this study.

3.2 *In vitro* biofilm model development and culturability monitoring

An *in vitro P. aeruginosa* biofilm model was developed in 24-wells microtiter plates and 3 different stress conditions (i.e. starvation alone or combined with fluoroquinolones/aminoglycosides antibiotics) were examined for their involvement in inducing the VBNC state. Culturability was assessed at different time points, searching for *P. aeruginosa* biofilms loss of culturability and/or metabolic changes (i.e. slow-growing colonies) and atypical phenotypes. The culturability results are reported in Fig. 9.

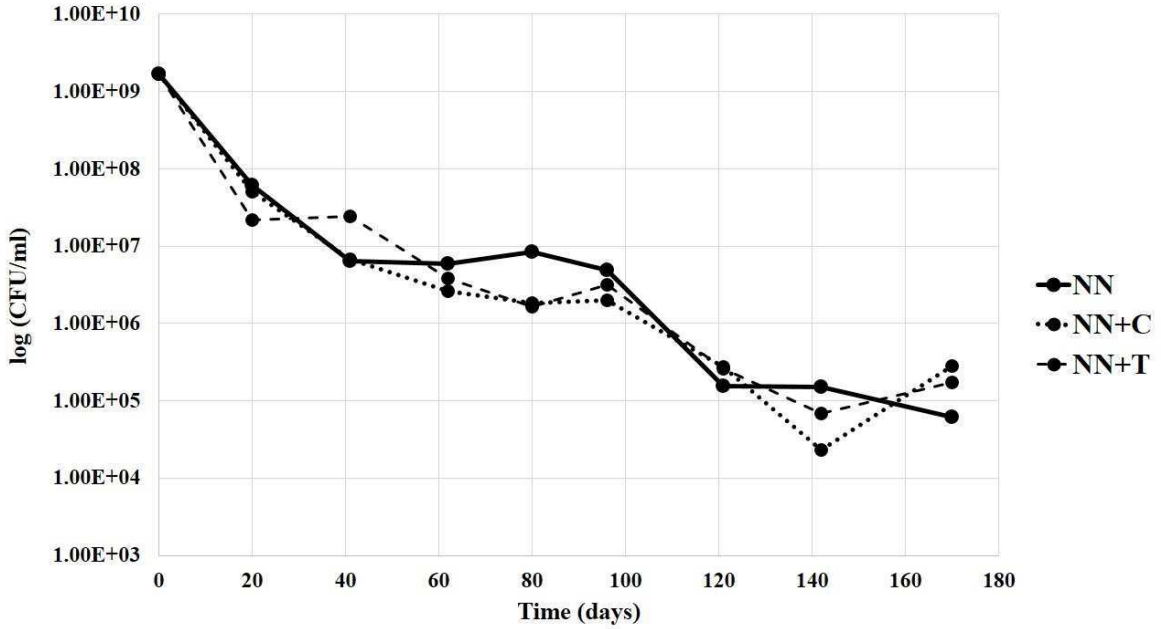

Fig. 9. Seventy-two hours cultural counts of *P. aeruginosa* C24 biofilms exposed to 3 stress conditions, i.e. starvation (NN), starvation plus ciprofloxacim (NN+C) and starvation plus tobramycin (NN+T). No significative difference was noticed between the 3 conditions, showing all a major decrease in the first 120 day of analysis.

As shown in Fig. 9, *P. aeruginosa* C24 biofilms did not loose their culturability in the analysed period of time in any tested condition, however they showed an uniform reduction (about 4 log) in the CFUs counts, with a more evident decrease in the first 120 days of the study.

These results are in agreement with two well-known features of *P. aeruginosa*: the great genome plasticity and the proneness to adaptat to different environmental conditions, evidenced by the general stability of the CFUs counts after the first 120 days (Stover et al., 2000). A further confirmation was provided by the insurgence of SCVs, starting from T40, observed in each stress condition, and by the recovery of swarming colonies, only observed in samples not exposed to antibiotics. The former case is a clear sign of the pathogen adaptation both to nutrient depletion and antibiotic pressure (Malone, 2015), whereas the latter confirms the influence of nutrient sources on *P. aeruginosa* motility and biofilm formation exposed in the "motility hypothesis"(Shrout et

al., 2006): it was, indeed, demonstrated that secondary carbon sources impact on the pathogen metabolism, promoting the swarming motility and forming a monolayer biofilm, compared to a more complex structure, typical of *P. aeruginosa* biofilms developed on glucose.

Previous studies performed on *S. aureus* had described the pathogen loss of culturability when exposed to stress conditions, especially antibiotic pressure (Pasquaroli et al., 2013); thus, we were surprised that *P. aeruginosa* C24 biofilms maintained their culturability, even when exposed for a long period (i.e. 170 days) to antibiotics. However, it is possible to speculate that a constant culturable bacterial population is maintained thanks to the programmed lysis and nutrient release from other cells (Lewis, 2000). This, in the case of a host infection, might contribute to the lung stable colonization and the antibiotic treatment failure.

Starting from T40 cultural counts performed after 72h incubation at 37°C resulted higher than those obtained after 24h and no CFU was detected after 24h incubation in agar plates containing tobramycin. In all the other conditions (i.e. NN and NN+ciprofloxacin) *P. aeruginosa* C24 CFU were detectable after 24h incubation, and the further 48h incubation caused an increase (between 0.5 and 1 log) of their number.

This might be due to the specific target of tobramycin action, i.e. protein synthesis, possibly determining a metabolism slow-down. However, it must be noticed that the antibiotic was present at a subinhibitory concentration (i.e. 1/4x MIC) and, furthermore, some intrinsic aminoglycoside resistance mechanisms have been described in *P. aeruginosa* (Morita et al., 2012), some of them even specific for the biofilm lifestyle (Beaudoin et al., 2012). Thus, it seems more appropriate to think about an expression modulation effect due to the low antibiotic concentration, i.e. hormesis, as described by Davies et al. (2006). The

possibility of another hormetic effect, specifically the induction of VBNC forms, was investigated.

3.3 *P. aeruginosa* dormant forms detection

3.3.1 Epifluorescence microscopy enumeration

The presence of VBNC forms in *P. aeruginosa* C24 biofilms exposed to stress conditions was assessed at specific time points by epifluorescence microscopy, after live/dead staining of the detached biofilms. Specifically, the amount of VBNC cells was obtained by comparing the counts of green stained cells with the CFUs counts performed at the same time points. The results are reported in Fig 10.

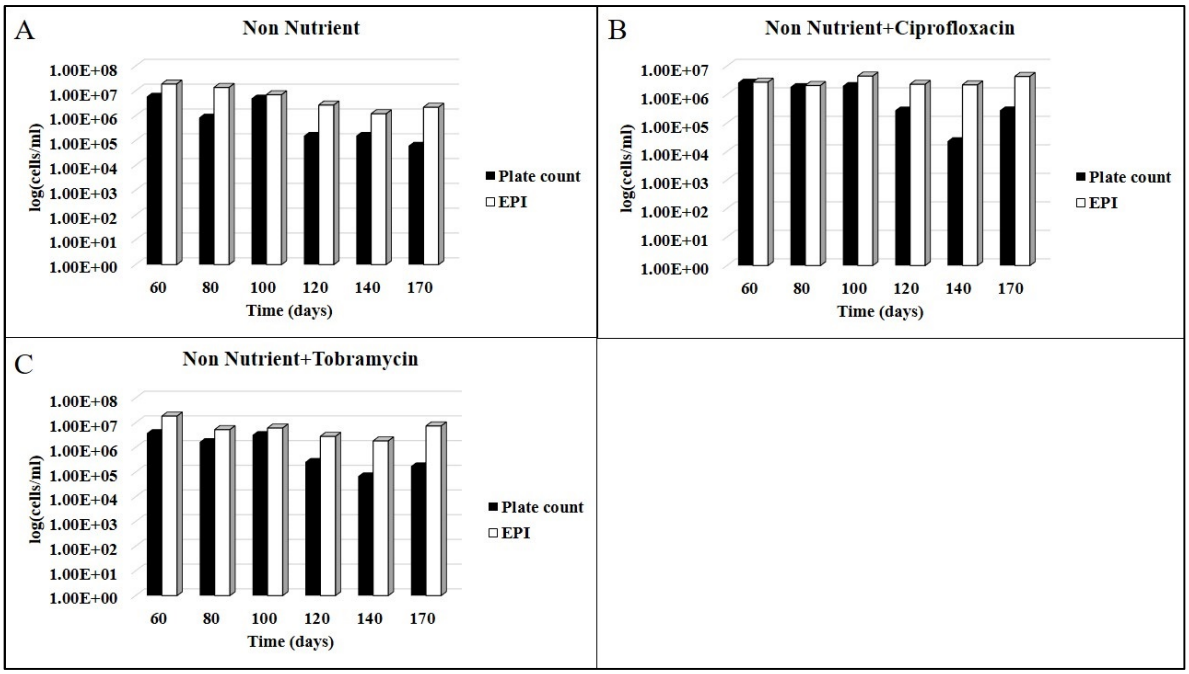

Fig. 10. Comparison of CFUs and epifluorescence microscopy (EPI) cell counts of *P. aeruginosa* C24 biofilms, exposed to starvation (A), starvation and ciprofloxacin (B) or starvation and tobramycin (C), at different time points.

Only in biofilms exposed to ciprofloxacin similar viable cell counts were observed in the first 100 days-incubation, whereas in biofilms exposed to both starvation alone or starvation and tobramycin epifluorescence microscopy counts were always higher than CFU counts.

This may suggest the presence of a VBNC population, not detected by cultural techniques. However, no significant difference was detected between the 3 stress conditions, suggesting the absence of correlation between the kind of stress and the presence of a viable unculturable subpopulation. Moreover, it must be taken in account that epifluorescence counts may be greatly affected by the conversion factor (i.e.31360). Thus epifluorescence microscopy, while proven to be a good technique for the detection of bacterial VBNC forms (Pasquaroli et al., 2013), in the presence of bacterial unculturability, seems otherwise a quite unaccurate method, to detect the amount of viable cells. Epifluorescence counts were thus just used as a further support of qPCR results in cases where they resulted higher than CFU counts (see below).

3.3.2 qPCR quantification

The *P. aeruginosa* counts in stress-exposed biofilms were further assessed by qPCR, using the *ecfX*-targeting protocol previously described for the analysis of CF sputum samples. The obtained results were compared with the CFUs counts performed at the same time points, (Fig. 11).

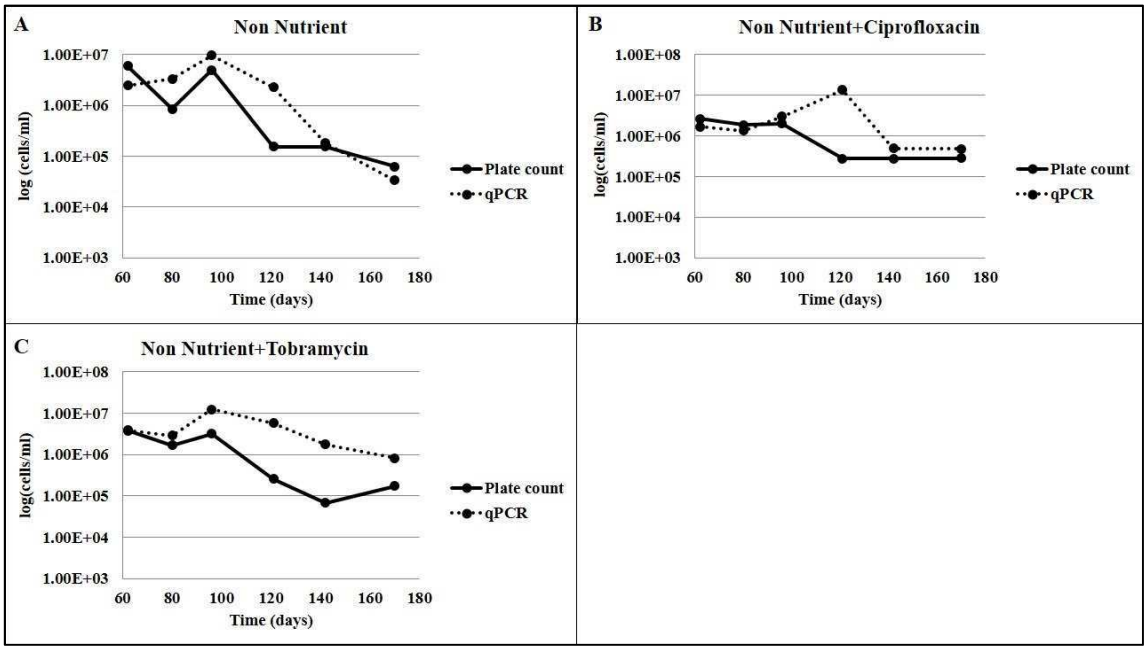

Fig. 11. Comparison of plate and qPCR counts of *P. aeruginosa* C24 biofilms exposed to starvation (A), starvation and ciprofloxacin (B) and starvation and tobramycin (C).

Only in tobramycin-exposed biofilms a consistent and persistent difference between the results obtained by the CFUs and qPCR counts was observed, suggesting the presence of a stable *P. aeruginosa* VBNC population. The presence of ciprofloxacin seemed to induce only a transient VBNC population, no more detectable at the end of the experiment.

qPCR counts revealed the presence of bacterial VBNC forms in *P. aeruginosa* biofilms, consistenly with the results obtained with CF sputum samples and gave new insights on possible inducers of the VBNC state. No significant difference between qPCR and plate count was detected in biofilms exposed only to

starvation, suggesting that this condition alone might be inadeguate to induce the dormant state. On the contrary, subinhibitory antibiotic concentrations seemed to be determinant for the development of the VBNC population. Different antibiotics can exert different results; in our case, the induction of VBNC forms was limited for ciprofloxacin, with an evident difference between CFUs and qPCR counts detected only between T100 and T140. These data are in agreement with previous observations (Jørgensen et al., 2013; Wassermann et al., 2016), describing a hormetic effect (i.e. phenotypic alterations) exerted by a long-term exposure to subinhibitory concentrations of ciprofloxacin in *P. aeruginosa* broth cultures. Nevertheless, the time lapse showing the presence of VBNC forms seems to be limited if compared to the whole lenght of the study and, furthermore, the dormant population seems to automatically disappear, without adding further perturbing conditions. These data suggest that while ciprofloxacin subinhibitory concentrations can be responsible for the induction of an unculturable *P. aeruginosa* population, this state might represent a pre-mortem condition, rather than a true VBNC state. The pre-mortem state has a different clinical significance compared to the VBNC state, as previously reported for *S. aureus* by Pasquaroli (2014).

A true, VBNC inducing role seemed to be exherted by tobramycin. Indeed, in tobramycin-exposed *P. aeruginosa* C24 biofilms from T80 to the end of the study, qPCR results were higher than CFU counts (Fig.12).

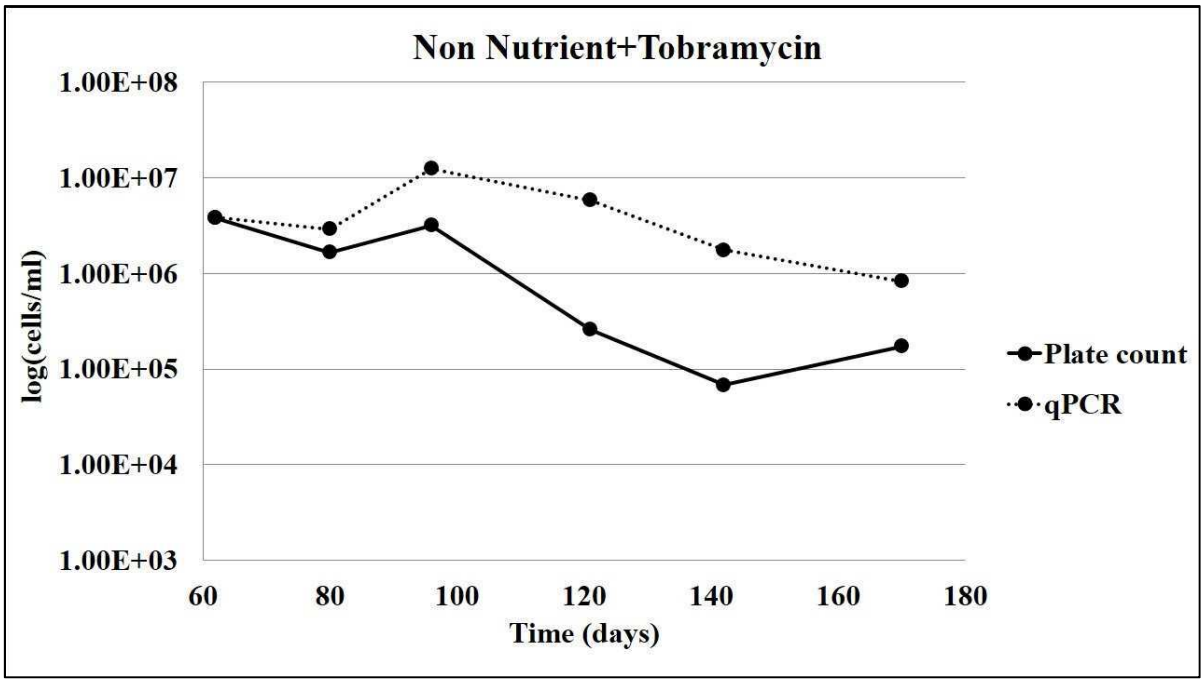

Fig.12. Comparison of plate counts and qPCR quantifications of *P. aeruginosa* C24 in biofilms exposed to starvation plus 1/4xMIC tobramycin.

The gap between qPCR and CFUs counts, quite small (<0.5log) at T80, consistently increased starting from T100. Since higher qPCR values due to presence of eDNA may be excluded due the previous validation of the DNA extraction protocol, the presence of a stable non-culturable subpopulation can be inferred. These data are supported by the phenotypical behaviour of the pathogen, showing only in presence of tobramycin a growth slow-down and the absence of culturability after 24h incubation at 37°C. The tobramycin mechanism of action, impairing the protein synthesis and causing metabolic damages, might be somehow correlated to the induction of the VBNC state, characterized by a very low metabolic activity (Oliver, 2005); on the other hand, the loweness (subinhibitory) of the antibiotic concentrations used in our experiments recalls a hormetic effect of antibiotics reported by Davies (2006). We can assume that the low antibiotic amount used has modulated the *P. aeruginosa* gene expression, leading to the induction of the VBNC state as stress response. This is in agreement with the results reported for *S. aureus* biofilms, that showed the development of VBNC forms after exposure to subinhibitory

concentrations of quinupristin/dalfopristin, a protein synthesis inhibitor (Pasquaroli et al., 2013).

Moreover, consistent information is provided by clinical management of both early and chronic CF *P. aeruginosa* infections, both routinely treated with tobramycin inhaled solutions (Ratjen et al., 2009). These formulations have proved to be very effective in reducing the bacterial load and in the delay of chronic infections (Stanojevic et al., 2014); however, the failure of the pathogen eradication, once *P. aeruginosa* colonises CF lung, might be explained by the induction by the antibiotic itself of *P. aeruginosa* VBNC forms, able to revert to the full metabolic, culturable state once antibiotic pressure is over.

4. CONCLUDING REMARKS

The induction and maintenance of *P. aeruginosa* VBNC forms could represent one of the main reasons of antibiotic treatment failure in CF pulmonary infections and the identification of their inducers is an important goal of microbiology research. The possibility that specific antibiotics, like tobramycin, can exert a survival rather than an inhibitory effect could give new insights in the management and development of new therapeutic protocols. Although from this study the hormetic effect of sub-MICs of tobramycin may be inferred, several features of this phenomenon are still to be understood. In particular:

- the dose-dependent effect of VBNC induction, testing different sub-inhibitory tobramycin concentrations;
- The involvement of efflux pumps in the convertion from full metabolic active to dormant cell form and in its maintenance;
- The specific features connonative of *P. aeruginosa* VBNC forms,;
- The stimuli responsible of resuscitation and its implication in the infections recurrency;
- The main factors able to riactivate the VBNC forms, with a particular focus on nutrients availability, oxidative stress protection and also the possible role of specific antibiotic concentrations.

CHAPTER III- GENETIC REGULATION OF *P. aeruginosa* PERSISTENCE

1. INTRODUCTION

1.1 Persistent bacterial cells

Persistent bacterial cells were described soon after the introduction of the intense use of antibiotics in the infections treatment (Lewis, 2010). They were first described by Bigger in 1944, who ricovered surviving *S. aureus* colonies after adding penicillin to bacterial cultures. They are defined as slow-growing cell forms, able to tolerate antibiotic action; this feature constitutes the main difference with the mutant strains, since in persister cells the antibiotic action is not prevented and they do not show a different genetic background from their parental strains.

The development of persister cells can be detected by antibiotic killing curves, after exposure of bacterial cultures to high doses of antibiotics. As shown in Fig. 13, it is characterized by a bifasic pattern, showing a typical plateau phase within few hours after exposure to the toxic substance.

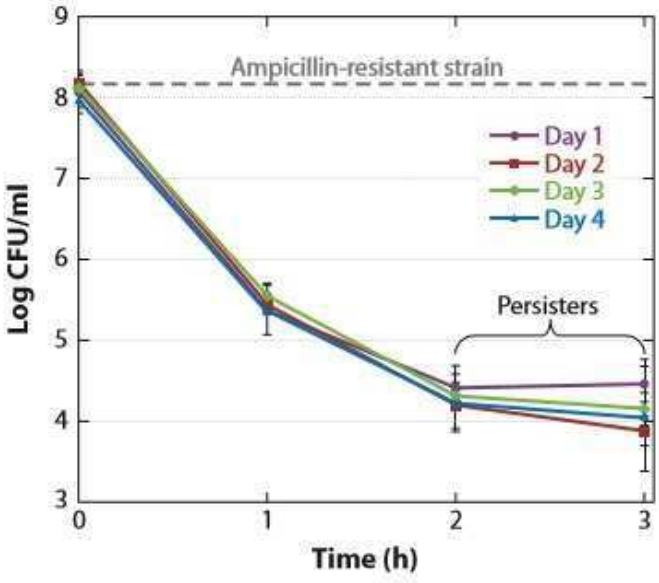

Fig. 13. Persistence assay performed on an exponential phase *E. coli* culture. The culture was subjected to high doses of ampicillin and persisters formation was assessed by plate-count method; persister cells were then cultured again and exposed to ampicillin for 4 consecutive days, repeating the assay. The typical plateau phase is evidenced between 2 and 3 h after antibiotic exposure. The persisters behaviour was compared to ampicillin-resistant mutants, represented by the dashed line.

The antibiotic tolerance is the most known feature of persisters; further characterizations are difficult to perform, given their low frequency and transient state (Hazan et al., 2014), especially in *P. aeruginosa* infections.

Although they are generally considerated a stochastic phenotype (Ayrapetyan et al., 2015), a great attention has been dedicated to the study of persister cells formation, in order to identify possible targets to develop an anti-persisters therapy. The main responsible factors reported are the toxin-antitoxin (TA) systems (Lewis, 2010); these are protein modules, encoded by both bacterial chromosome and plasmids, responsible for the population maintenance, usually inherited by daughter cells during bacterial division. The toxin results to be stable, whereas the antoxin presents a degredable nature; according to this model, the repression/loss of the antitoxin allows the toxin effect, often targeting a metabolic pathway, and can induce a slow-growing phenotype. This one is responsible for the antibiotic tolerance, since most antibiotic effects rely on an intense metabolic activity and lead to cell death disrupting a crucial pathway. When metabolism is slown-down, antibiotics cannot fully achieve their effect.

Among the various described *P. aeruginosa* TA systems, the most important reported are the HipAB and TisAB systems. The former is the first gene-module related to persistence and its name is due to its presence in “high-persistence mutants” (Lewis, 2012); the HipA toxin is a protein kinase, able to autophosphorilate itself and targeting the elongation factor Ef-Tu, thus impairing protein translation and methabolism.

The second one acts on a more general pathway: the TisB toxin forms a pore in the bacterial cytoplasmic membrane, decreasing the proton-motife force involved in ATP synthesis and depleting the cell metabolic resources.

Other general mechanisms have been proposed to contribute to bacterial persistence: in *P. aeruginosa*, the involvement of pyocianin and, most of all, of signal molecules, such as the quorum sensing homoserine lactones, have been

reported to increase the percentage of persisters in a species-specifc way (Möker et al., 2010).

The involvement of persister cells in recurrent and chronic bacterial infections has been demonstrated. They have been mostly associated to biofilm-related infections, since biofilm is considered a protective environment for persister survival, in particular for the CF *P. aeruginosa* pulmonary infections. Indeed, it has been shown that, among longitudinal CF *P. aeruginosa* isolates recovered from the same patients, the late ones present higher persisters formation, despite showing the same antibiotic MIC values of the early isolates (Mulcahy et al., 2010). Given the high doses of antibiotic used in antibiotic treatments of pulmonary exacerbations, it is not surprising that a persister subpopulation can be selected and, even thanks to the protection provided by biofilm, ripopulate the CF lung.

Although these mechanisms have been well elucidated, microbiologists are still searching for genetic pathways responsible for persister cells formation, in order to clarify the connection between environmental stimuli, including the presence of toxic compounds, and persisters development.

1.2 The Gac/Rsm system

The Gac/Rsm system is a well characterized and conserved transducing signal pathway in the genus *Pseudomonas* and results involved in several genetic, metabolic and phenotypic pathways of *P. aeruginosa* (Frangipiani et al. 2014). It is composed by the two-components system GacS/A, a sensor kinase and an effector protein, and by the RsmA protein (Coggan and Wolfgang, 2012). The sensor kinase responds to environmental stimuli by phosphorilating the GacA protein that controls the expression of the small untranslated RNAs (sRNA) RsmY and RsmZ; these two bind and sequester the RsmA protein, a post-transcriptional regulator, affecting both negatively and positively the expression of a wide range of genes. The whole system acts in a coordinate way along with

other *P. aeruginosa* signal pathways and is deeply influenced by other signal molecules. The most known are the sensor kinase/response regulator RetS and the second messenger c-di-GMP. The former acts directly on the GacS/A system, inhibiting the activation of the effector protein and allowing the RsmA action (Goodman et al., 2004). The second messenger c-di-GMP has been described as regulator of the same processes of the RsmA protein, but with an antagonist effect (Colley et al., 2016).

The Gac/Rsm system is extremely important in the switch between planktonic and sessile lifestyle and, referring to human infections, between acute and chronic stages (Goodman et al., 2004). During acute infections, the RsmA protein is free and available in the bacterial cells and it positively controls the expression of different virulence factors (i.e. type IV pili, T3SS, ToxA and LipA), whereas it represses the production of exopolysaccharides and quorum sensing signal molecules. When a chronic infection is developed, the effector protein GacA is activated and mediates the sequestering of RsmA, allowing the expression of various factors involved in cell-to-cell aggregation and biofilm formation.

Since it has been described that persister cells are favored in survival by biofilm formation, it could be speculated that the action of the GacS/A system and the loss of action of the RsmA protein may contribute to an increase of persister cells production, or that they still play a role in this phenomenon.

The RsmA protein has been described as well conserved in the *Pseudomonas* genus and different homologue proteins have been reported (Morris et al.,2013); one of these in particular, RsmN, is *P. aeruginosa* species-specific. Moreover, although presenting a different protein structure and conformation compared to RsmA, it seems to recognize most of its target-mRNAs, suggesting overlapping roles of the two proteins and a redundant control mechanism of the same *P. aeruginosa* pathways.

1.3 The second messenger c-di-GMP

The c-di-GMP is synthesized from 2 molecules of GTP through the activity of diguanylate cyclases and it is degraded by phosphodiesterases. It acts like second messenger, regulating a wide variety of metabolic pathways. In *P. aeruginosa*, it is particularly involved in biofilm formation (Ha and O'Toole, 2015). Indeed, biofilms are characterized by higher c-di-GMP levels compared to planktonic cultures and the second messenger has resulted affecting almost every step of the biofilm formation process:

- Flagellar synthesis, implicated in the initial reversible adhesion, through FleQ expression control;
- Cell-to-cell adhesion, microcolonies formation and biofilm development by controlling the CdrA protein;
- Pel exopolysaccharide production inducing the *pelD* gene expression;
- Bacterial dispersal from biofilm (associated with decreased c-di-GMP levels).

c-di-GMP has also been described as regulator of bacterial motility, stress conditions adaptation and even in the induction of SCVs (Coggan and Wolfgang, 2012).

As the Gac/Rsm system, it is involved in the switch between motile and sessile lifestyle; according to the model proposed by Li K. et al. (2017), the SuhB regulator controls the reversible convertion from *P. aeruginosa* planktonic cells to sessile ones by activating/repressing the Gac/Rsm system and modulating c-di-GMP levels, increasing the RsmA or c-di-GMP levels in association to planktonic lifestyle or biofilm one respectively.

Interestingly, it has been demonstrated that the artificial modulation of the second messenger levels is sufficient to induce the convertion of phenotypes typical of acute infections to those recovered during chronic infections, including the switch of expression from T3SS to T6SS (Moscoso et al., 2012).

The evidences of the antagonism RsmA/c-di-GMP and the involvement of the latter in SCVs development suggest that even this second messenger could be involved in persistence formation, especially during biofilm growth.

1.4 Aims of the study

The aim of this study, performed at the laboratory of Professor Paul Williams, Centre for Biomolecular Sciences, University of Nottingham, and based on previous results about *P. aeruginosa* persistence, was to identify the main genetic pathways involved in persister cell induction in *P. aeruginosa*. The previously obtained data regarded the identification of the TA modules affecting persisters formation and the possible involvement of known regulatory pathways in the control of *P. aeruginosa* persistence. In this study, the attention was mainly focused on the Gac/Rsm system, in particular on the RsmA protein, since regulating the switch between acute and chronic infection and the related biofilm production. Its involvement in *P. aeruginosa* persistence, along with its homologue protein RsmN, was assessed in bacterial biofilm, testing the activity of the two most used antibiotic in *P. aeruginosa* infections treatment, i.e. ciprofloxacin and tobramycin.

As further investigation, we assessed the role of the second messenger c-di-GMP, known often to antagonize RsmA activity, by measuring its levels in antibiotic-exposed biofilms.

2. MATERIAL AND METHODS

2.1 Bacterial strains

The *P. aeruginosa* strains were provided by the Professor Paul Williams Quorum Sensing Research Group, Centre for Biomolecular Sciences, University of Nottingham and are listed in Tab. 3.

Table 3. Bacterial strains involved in the study

STRAIN	DESCRIPTION
PAO1-N	*P. aeruginosa* PAO1 wild type, Nottingham subline
PAZH13	*P. aeruginosa* PAO1-N Δ*rsmA*
PALT16	*P. aeruginosa* PAO1-N Δ*rsmN*
PALT13	*P. aeruginosa* PAO1-N *rsmN*ind Δ*rsmA*
PAO1-N+p*cdrA*+gfp^s	*P. aeruginosa* PAO1-N expressing the *gfp* gene under the control of the *cdrA* promoter
PAZH13+p*cdrA*+gfp^s	*P. aeruginosa* PAZH13 expressing the *gfp* gene under the control of the *cdrA* promoter
ATCC 27853	*P. aeruginosa* American Type Culture Collection 27853 wild type

The *P. aeruginosa* mutant strains were obtained by allelic replacement of the gene of interest with a suicide plasmid, carrying the in-frame deletion of the same gene, transferred in *P. aeruginosa* PAO1-N by conjugation.

The p*cdrA*+gfp^s plasmid was transferred into *P. aeruginosa* PAO1-N by electroporation, using 2.5 kV pulses in pre-chilled electroporation cuvettes.

2.2 MIC determination

Ciprofloxacin and tobramycin MICs against *P. aeruginosa* PAO1-N and its isogenic mutants for the *rsmA* and *rsmN* genes (i.e. PAZH13, PALT16 and PALT13) were determined following the agar dilution method procedure, as reported in CLSI guidelines (2017). Briefly, *P. aeruginosa* strains were grown overnight in MH broth at 37°C, with shaking (200 r.p.m) and then diluted till reaching an OD_{625}=0.1. Five µl inocula were then spotted on MH agar plates unsupplemented/supplemented with different antibiotic concentrations, ranging from 0.06 to 4 µg/ml. After 24h incubation at 37°C, bacterial growth was evaluated and the antibiotic concentration reporting no growth on plate was considered the antibiotic MIC. *P. aeruginosa* ATCC 27853 was included as reference strain.

2.3 Biofilm production assay

Since *P. aeruginosa rsmA*-mutants (i.e. PAZH13 and PALT13) exhibited the formation of aggregates during planktonic growth (data not shown), the ability of forming biofilm was examined in *P. aeruginosa* PAO1-N and its isogenic mutants for *rsmA* and *rsmN* genes, in order to elucidate the impact of the 2 selected targets on the sessile growth.

Briefly, bacterial strains were grown overnight in 5 ml Luria Bertani (LB) broth at 37°C, with shaking (200 r.p.m.) and then diluted till reaching an OD_{600}=0.05. Two ml aliquots of the diluted cultures were inoculated into sterile 15 ml borosilicate glass tubes and put in rotation with around 45° inclination at 30°C for 7 h, to allow biofilm formation. Planktonic cultures were then removed and their OD_{600} measured; tubes were washed with 5 ml of water and stained with crystal violet 1% for 15 min. Tubes were then washed again and crystal violet removed using acetic acid 30%; crystal violet was then diluted 1:50 in acetic acid 30% and the OD_{570} was read. The obtained values were normalized, dividing them for the OD_{600} of the relative removed culture. *P. aeruginosa*

PAO1-N was considered the reference strains (i.e. biofilm production 100%) and used to compare the remaining strains. Each strain was tested two times in triplicate.

2.4 Persistence assays

The ability of *P. aeruginosa* PAO1-N and its isogenic *rsmA/N*-mutants to develop persister cell forms after exposure to ciprofloxacin or tobramycin was investigated in bacterial biofilms, in order to identify the genetic backgroud mainly responsible for their induction.

Bacterial biofilms were formed in sterile 15 ml borosilicate glass tubes, as described above. After 7h incubation in rotation at 30°C, tubes were washed twice with 5 ml PBS, additioned with 2 ml *Pseudomonas* Isolation (PI) broth, supplemented with antibiotic at a concentration of 1000xMIC and put again in rotation. Immediately after antibiotic exposure and after further 1, 3, 5 and 24 hours, one tube for each strain was collected, washed twice with PBS and resuspended in 1 ml PBS. Biofilm was detached mechanically from the tube sides by adding 3 glass beads and vortexing them at low speed. The resuspended biofilm was then collected and assessed as follows:

- CFUs count: biofilm was serially diluted in PI broth and 100 µl aliquots were inoculated on LB agar plates. CFUs count was performed after 24h incubation at 37°C.
- Luminescence: the metabolic state of antibiotic exposed cells in biofilm was assessed measuring the ATP levels (luminescence, RLUs) in resuspended biofilm, using the BacTiter-GLO™ Microbial Cell Viability Assay kit (Promega Corporation, Madison, Wisconsin, USA). Briefly, 100 µl of resuspended biofilm were transferred to a 96-wells-black-flat microtiter plate and added with 100 µl of BacTiter-GLO reagent. The plate was shaken at 150 r.p.m. for 5 min and then luminescence (attenuation none,

integration time 1 s) was read using Infinite 200 plate reader (Tecan, Männedorf, Switzerland).

The assay was performed on *P. aeruginosa* PAO1-N and its isogenic rsmA/N mutants biofilms; each strain was tested two times in triplicate.

2.5 c-di-GMP level measurement in bacterial biofilms

To assess the involvement of c-di-GMP in *P. aeruginosa* persistence, the second messenger levels were measured during a ciprofloxacin persistence assays performed on *P. aeruginosa* PAO1-N and PAZH13 biofilms. The 2 strains were implemented by the p*cdrA*+gfp plasmid, expressing the Green Fluorescent Protein (GFP) under the control of the *cdr*A promoter, responding to c-di-GMP presence. Since the plasmid is maintained through growing the strains in LB broth additioned with gentamycin 10 µg/ml, this assay was not performed with tobramycin, to avoid possible interferences. Biofilms were grown as described above and processed as in a persistence assay. The same biofilms, not exposed to ciprofloxacin, were processed as well, as control. After detachment from the tube sides, 200 µl of resuspended biofilms were transferred to a 96-wells-flat clear bottom-black-microtiter plate and read at Infinite 200 plate reader (Tecan). Biofilms OD_{600} and fluorescence intensity, given by the GFP in response to c-di-GMP levels (absorbance 485 nm, emission 535 nm), were read, then fluorescence was normalized dividing it for the related OD_{600}. Each strain was tested in triplicate.

3. RESULTS AND DISCUSSION

3.1 *P. aeruginosa* strains characterization

In order to evaluate the influence of the *rsmA/N* genes in *P. aeruginosa* persistence, the wildtype PAO1-N strain and its isogenic mutants for the two target genes were characterized for antibiotic resistance and biofilm formation. The first evaluation, in particular, was meant to exclude the potentiation of some resistance mechanisms, that would have impaired the subsequented analyses and would not have been respondent to the definition of persister cell as antibiotic tolerant.

The MIC assays results are reported in Tab. 4.

Table 4. Ciprofloxacin and tobramycin MIC values for *P. aeruginosa* PAO1-N and RsmA/N mutants

	MIC (µg/ml)			
STRAIN	PAO1-N	PALT16	PAZH13	PALT13
CIPROFLOXACIN	0.25	0.25	0.25	0.25
TOBRAMYCIN	1	1	1	1

The loss of either RsmA (i.e. PAZH13) or RsmN (i.e. PALT16) or both (i.e. PALT13) did not affect MIC values; this indicates that Gac/Rsm does not control antibiotic resistance mechanisms, at least not directly. Indeed, although mutations in this regulatory pathway and in the regulator genes of intrinsic resistance determinants have been described as both typical of adapted genotypes of chronic infections (Marvig et al., 2015), no direct connection has been reported. Thus, any difference in CFUs counts during persister assays can be surely attributed to a different persister cells induction.

On the contrary, biofilm formation resulted higly influenced by RsmA/N presence, as shown in Fig. 14.

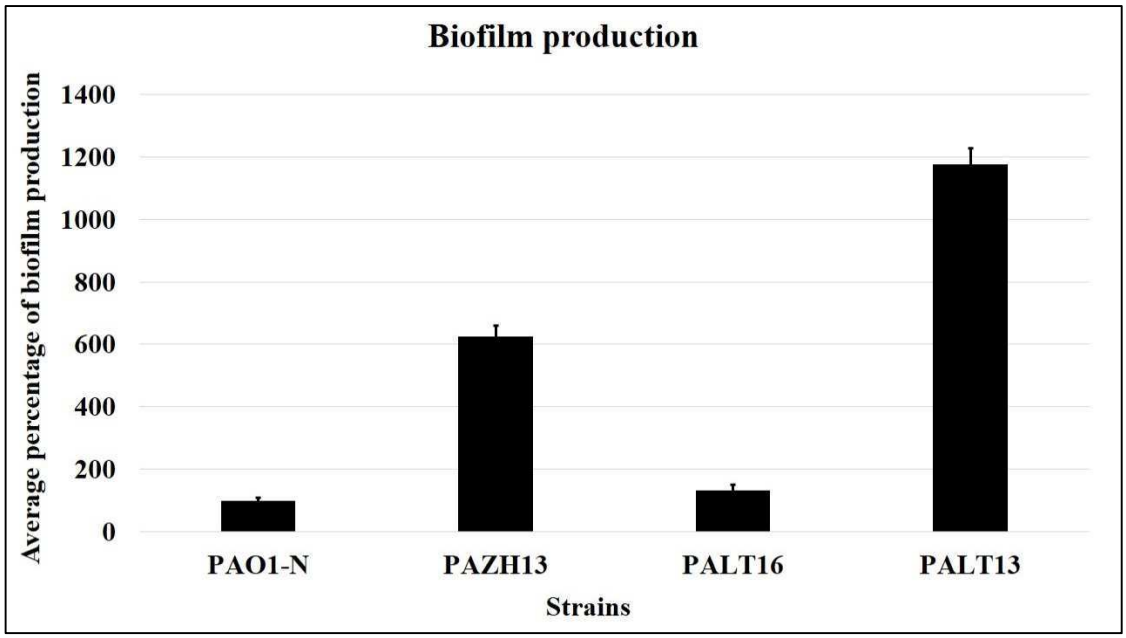

Fig. 14. Biofilm production of *P. aeruginosa* PAO1-N and its isogenic mutants for *rsmA/N* genes. Biofilm formation was normalized considering the optical density of the planktonic culture removed by biofilm samples and resulted 6 and 12 times higher in *rsmA*-single and *rsmA/N*-double mutants respectively, in comparison to the wild-type strain.

Biofilm formation resulted increased in absence of the *rsmA* gene (i.e. *P. aeruginosa* PAZH13), wherease no difference was detected in absence of the *rsmN* gene alone (i.e. *P. aeruginosa* PALT16); the absence of both the genes caused a further increase of biofilm production, as seen for the double mutant *P. aeruginosa* PALT13 that showed a biofilm formation 12 times higher than the wild-type strain. These results on one hand confirm the data observed in literature, that report the RsmA protein involved in the expression of acute infection virulence factors and in the repression of exopolysaccharide synthesis; on the other hand, it is underlined that, although they can present overlapping roles and mechanisms, the RsmA and RsmN regulators are organized in a precise hierarchic scheme, where RsmN plays only a minor and redundant role.

3.2 Persistence assays

P. eruginosa biofilms persistence was evaluated, expecting a difference in persisters induction due to the absence of RsmA/N and using ciprofloxacin and tobramycin at a concentration of 1000xMIC (i.e. 250 and 1000 µg/ml respectively).

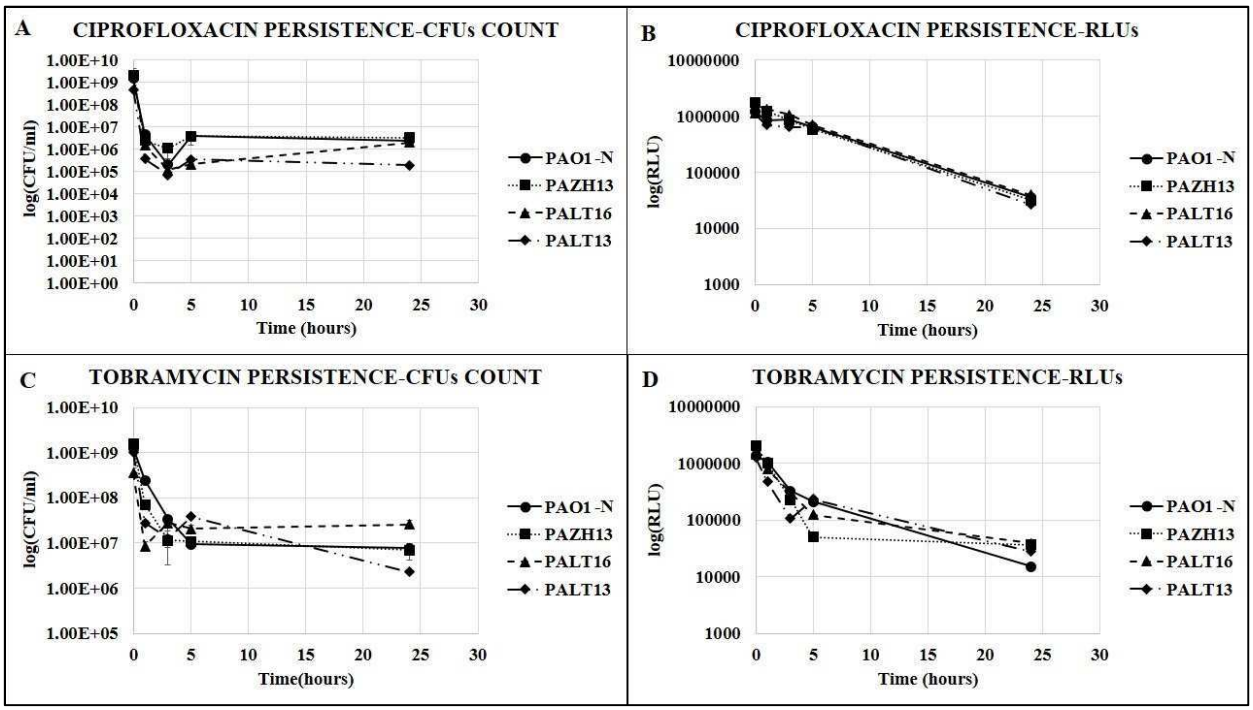

Fig. 15. Persistence assays performed on *P. aeruginosa* biofilms. Biofilms persistence in the presence of 250 µg/ml of ciprofloxacin and 1000 µg/ml of tobramycin (1000xMIC). Both CFUs counts (A, C respectively) and metabolic activity (B, D respectively) were evaluated. Metabolic activity was represented as luminescence emission in a viability assay.

As shown in Fig. 15, it was not possible to detect an overall significant difference in the amount of persisters. The only discrepancy from the wild type strain was observed against tobramycin, when evaluating the CFUs amount 1 hour after the antibiotic exposure (Fig 15 C), which resulted lower for the *P. aeruginosa* mutants compared to the wild-type. This difference is lost at the following time points. Indeed, while mutant cells are killed more quicly during the first hour of antibiotic exposure, their number remained stable till the end of the experiment; however their persister cells seemed to be selected by antibiotic exposure more directly than the wild-type, suggesting a faster and more efficient

adaptation to the stress condition. However, even in terms of metabolic answer, no significant difference was seen between the tested strains. Since both RsmA and RsmN are involved in the expression of virulence factors of the acute infection, their absence, mimicking their sequestering due to GacA activation, had been expected to cause a perturbation in the normal number of induced persister cells. These data don't seem to support the expected correlation between the activity of the post-trascriptional regulators RsmA/N and persisters formation. The involvement of redundant mechanisms able to complement the absence of the two regulators might be supposed. Considering that different regulatory pathways have shown to affect *P. aeruginosa* lifestyles switch (Moradali et al., 2017), the dectection of a RsmA-compensatory system would be not surprising.

3.3 Role of different antibiotics on persisters induction

From the data obtained in persistence assays it was still possible to evaluate the different response of *P. aeruginosa* to different antibiotics in terms of persister's formation.

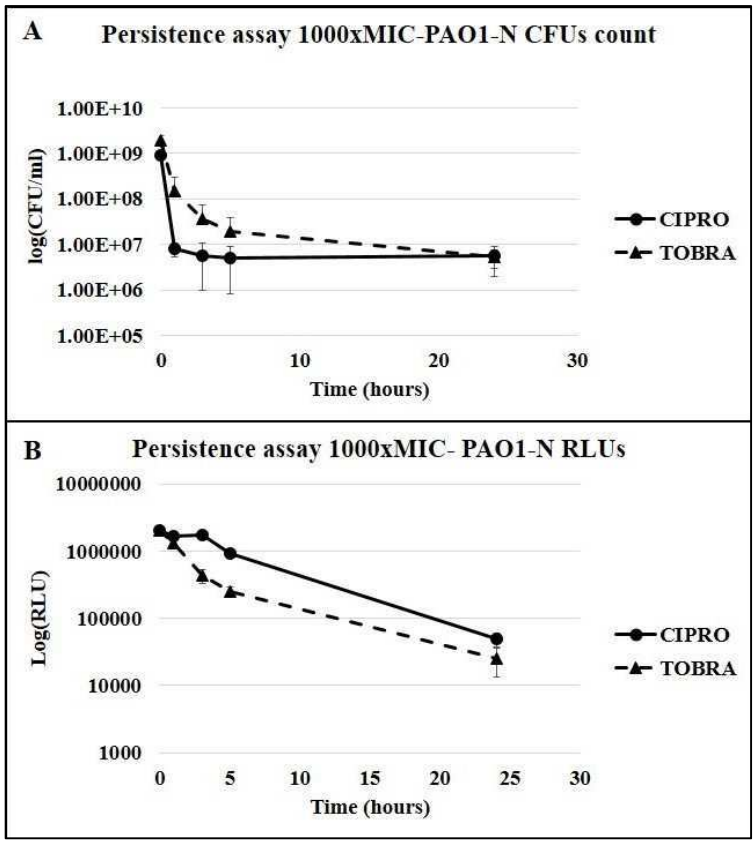

Fig. 16. Persistence assays performed on *P. aeruginosa* PAO1-N. Persisters formation was evaluated testing ciprofloxacin and tobramycin against *P. aeruginosa* PAO1-N biofilms, by CFUs counts (A) and measuring the metabolic response in terms of produced ATP, detected through a viability assay (B).

The wild-type strain *P. aeruginosa* PAO1-N showed different effects of the two antibiotics, as shown in Fig.16. Ciprofloxacin determined a faster and dramatic decrease of CFUs counts, already detectable after 1h exposure; however, in the following time points, the persisters level remained stable until the end of the experiment. The ATP levels, detected by luminescence measurement, showed a high metabolic level of *P. aeruginosa* in the first hours of antibiotic exposure, even considering the CFUs count decrease, whereas from 5 to 24 h ATP production progressively decreased, accordingly with the survival of the persister bacterial population only.

On the contrary, tobramycin exposure resulted in a lower and more gradual reduction of CFUs counts, in agreement with the observed decrease of ATP production. Furthermore, biofilms killing curves do not seem to reach a final plateau, but rather to continuously decrease, suggesting the instability of the

persistent subpopulation. These data seem specular to the ones obtained with VBNC forms induction (Chapter 2-Results and discussion-qPCR quantification). Ciprofloxacin can induce a persister subpopulation stable over time, which however in antibiotic-exposed biofilms recalls more a pre-mortem rather than a persistance state; tobramycin shows the opposite effect, giving rise to a persister's population, including VBNC forms, variable over time. If the hypothesis of these two phenotypes representing different features of the same dormant cycle is true (Ayrapetyan et al., 2015), it is possible that different antibiotics, attacking different microbial targets, can favor the induction of different stages of dormancy; indeed, the presence of different subpopulations can result crucial in a hostile environment, such as the lungs of CF patients, characterized by the costant presence of antibiotic pressure as a consequence of the therapeutic treatments.

In the present work *P. aeruginosa* cells persistence was evaluated mainly in terms of CFUs; it would be interesting to assess not only the ability to survive to high antibiotic doses, but also the ability to develop dormant forms (i.e.VBNC forms) in response to the treatment with different classes of antibiotics.

3.4 *P. aeruginosa* persistence and c-di-GMP

Since the presence of RsmA/N did not seem to impact on *P. aeruginosa* persistence, the effect of the second messenger c-di-GMP modulation was assessed by measuring its levels in biofilms of *P. aeruginosa* PAO1-N and its isogenic mutant *rsmA*-mutant (i.e. *P. aeruginosa* PAZH13) exposed to ciprofloxacin.

P. aeruginosa PAO1-N and PAZH13 were modified by adding the p*cdrA*+gfp plasmid, carrying the gene coding for the GFP under the *cdrA* promoter, induced by c-di-GMP. Biofilms of both strains were then analysed for the level of c-di-GMP in presence/absence of 250 µg/ml (1000xMIC) ciprofloxacin.

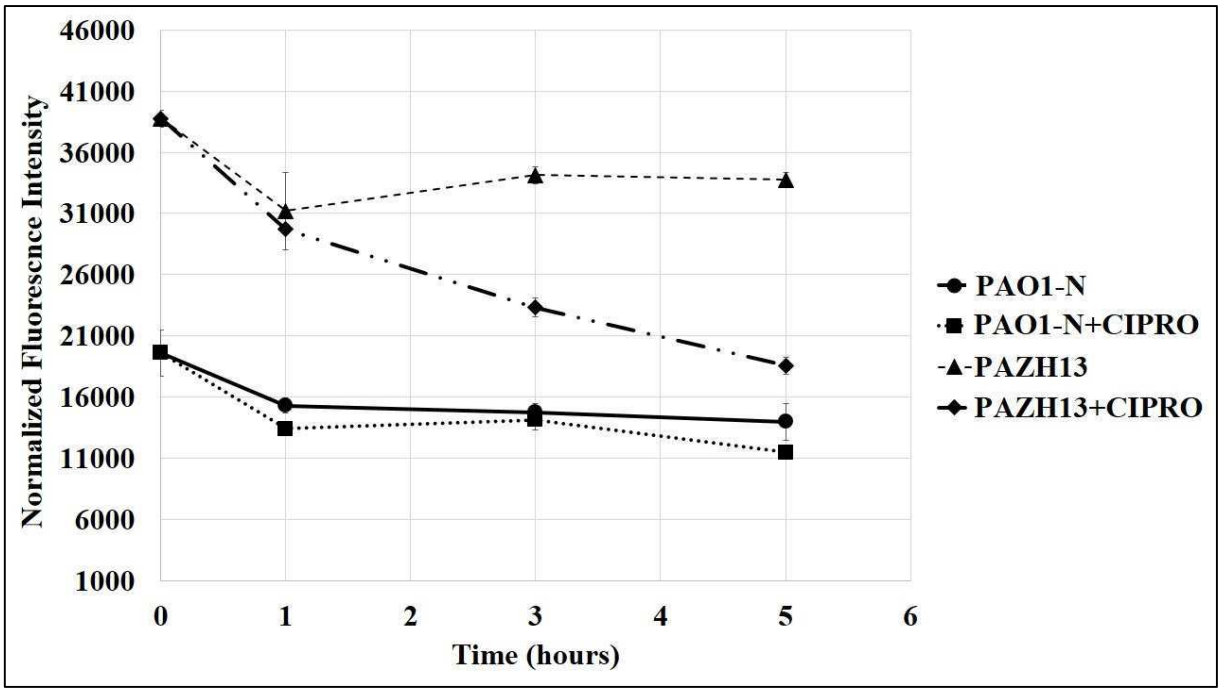

Fig. 17. c-di-GMP levels detection through fluorescence measurement in *P. aeruginosa* biofilms in presence/absence of 250 µg/ml ciprofloxacin (+CIPRO). An evident decrement of c-di-GMP levels was observed in *P. aeruginosa* PAZH13 biofilms when exposed to antibiotics, reaching the wild-type strains c-di-GMP levels.

As reported in Fig. 17, the antibiotic-free biofilms showed only a light reduction of c-di-GMP levels in the first hour of antibiotic exposure, stabilizing them at a constant value till its end. *P. aeruginosa* PAZH13 reported higher fluorescence values, as expected since producing more biofilm than the wild-type strain (Ha and O'Toole, 2015). The obtained values are even normalized by the OD_{600} of the planktonic culture grown along with biofilm, so no difference could be attributed to the different numbers of *P. aeruginosa* cells present at every time point. When ciprofloxacin was added to biofilms, the two strains exhibited different behaviours: *P. eruginosa* PAO1-N did not show any significant change in c-di-GMP levels compared to the antibiotic-free condition, maintaining constant fluorescence values from 1 to 5h of antibiotic exposure. On the other hand, in the presence of ciprofloxacin, *P. aeruginosa* PAZH13 c-di-GMP levels remarkably decreased for all the length of the experiment; interestingly, the final fluorescence value was comparable to the one obtained with *P. aeruginosa* PAO1-N.

The causes of this sudden and dramatic decrement are still being investigated; more interestingly, the c-di-GMP levels of the the wild-type strain do not vary upon antibiotic pressure, as if they represent a basic threshold, necessary to the *P. aeruginosa* population to assure the persisters formation. Consistent with this, c-di-GMP levels are down-regulated in *P. aeruginosa* PAZH13 biofilms exposed to antibiotic, and progressively decreased till reaching the levels of the wild-type strain PAO1-N. Although c-di-GMP has been more connected with SCVs formation (Malone et al., 2012), it is possible, since multiple regulator pathways result cross-linked in bacterial biofilms (Coggan and Wolfgang, 2012), that a protein effector is regulated by the second messenger levels and, in presence of low messenger concentrations, results crucial in persister formation.
Another hypothesis is that a protein effector is responsible for the resemblance of the c-di-GMP levels in *P. aeruginosa* PAO1-N and PAZH13 biofilms when exposed to antibiotics; since the only difference between the two strain is the RsmA absence, it looks possible that RsmN, showing overlapping effects with RsmA itself (Morris et al., 2013), could be involved in c-di-GMP downregulation. If the relationship second messenger-persisters is confirmed, this could even explain the absence of a significant difference in the behaviour between the wild type and the different mutants observed in the persistence assays. Moreover, the presence of alternative phosphodiesterases and diguanylate cyclases, responding to the antibiotic stimulus and regulating the messenger availability, could be involved in the control of persisters formation. *P. aeruginosa* presents several genes coding for enzymes regulating c-di-GMP levels (Leoni et al., 2017); so the presence of redundant regulatory patterns is not surprising.

4. CONCLUDING REMARKS

P. aeruginosa persistence represents a crucial problem in CF pulmonary infection treatment, since it allows the survival of bacterial cells and the infection recurrency.

This study provides first preliminary results about the identification of general regulatory pathways, probably involved in the induction of the persistent phenotype; the Gac/Rsm system has proved not to directly affect *P. aeruginosa* persistence formation, since no influence on the persisters amount was evidenced for the RsmA/N protein presence/absence. On the other hand, the second messenger c-di-GMP has provided some interesting results regarding its influence on persisters formation in *P. aeruginosa* biofilms.

Both targets are involved in a complex network of regulatory signals, controlling different features of the pathogen, and mainly the switch between sessile and planktonic lifestyle; thus, the interference of other molecules, or enzymes, playing compensatory roles looks like.

Further investigations will be dedicated to identify genetic pathways controlled both by the Gac/Rsm system and c-di-GMP, in order to evidence a common effect played on *P. aeruginosa* antibiotic persistence.

Moreover, other putative regulator pathways and molecules will be screened and characterized, to increase the understanding of the deep net of signals and communication within and between cells, in order to indentify promising targets for the developement of an anti-persister infection treatment.

CHAPTER IV- SEARCH FOR NATURAL EFFLUX PUMP INHIBITORS (EPIs) TO CONTRAST *P. aeruginosa* ANTIBIOTIC RESISTANCE

1. INTRODUCTION

1.1 *P. aeruginosa* RND EFFLUX PUMPS

P. aeruginosa RND efflux pumps represent the main intrisic antibiotic resistance mechanism described in this pathogen. Four main protein complexes belong to this class, specifically MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. As already cited, they all share the following composition (Ruggerone et al., 2013):

- A cytoplasmic transmembrane protein transporter, able to bind the antibiotic molecules and to expel them directly outside the bacterial cell, thanks to the proton motive force derived from the entrance of H^+ protons into the cell;
- A porin, located in the outer membrane, that allows the final molecule expulsion;
- A periplasmic protein that binds the other components and allows the formation of a functional complex.

The whole efflux mechanism relies on the action of the transmembrane transporter, whose activity has been well described in the RND transporter AcrB, the homologue of the MexB protein in *E.coli* (Murakami, 2008). It is present as a homotrimer and each monomer assumes a different conformation, related to the three stages of the efflux mechanism:

- Loose: the first step, it respresents the “entrance” for the antibiotic molecules in the efflux system, through the carboxy-terminal domain of the monomer;
- Tight: the bond between the protein and the antibiotic is stable and determines a structural modification of the complex;
- Open: the final stage, where the antibiotic is moved towards the external porin channel.

The efflux dynamics are shown in Fig. 18.

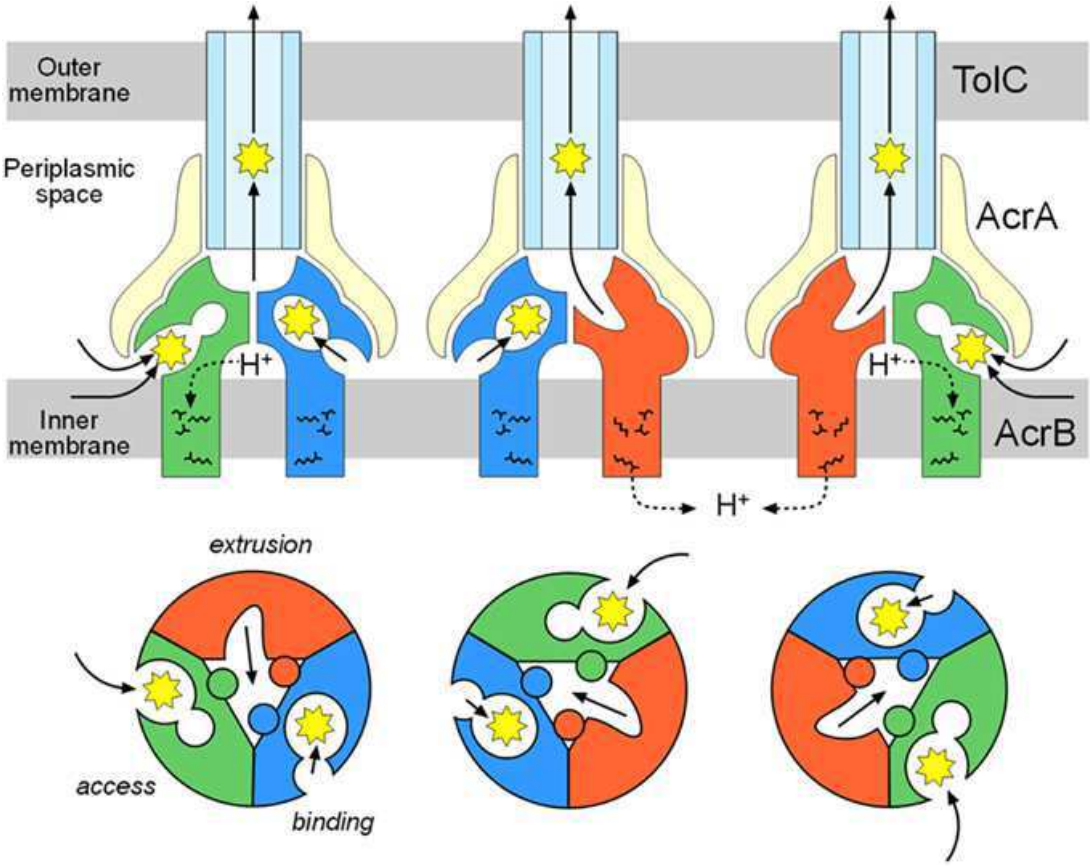

Fig. 18. Schematic representation of the AcrB monomers modifications during the efflux dynamics. The three monomers are represented in their three stages while binding and expelling the antibiotic, i.e. loose (access), tight (binding) and open (extrusion).

The three monomers form an internal cavity that allows the passage of expelled molecules and during the process they undergo a continuous conformational modification, characterized by rotation, causing a continuous and efficient efflux to the external environment. The same mechanism has been characterized for the MexAB-OprM efflux pump in *P. aeruginosa* (Sennhauser et al., 2009).

Moreover, the presence of a periplasmic cleft, helping the sequestering of antibiotics in the periplasmic space has been described (Lau et al., 2014).

The importance of these efflux pumps is due to their role in *P. aeruginosa* antibiotic resistance. Each one of them can extrude different antibiotics, resulting a typical defense in MDR and XDR *P. aeruginosa* strains and showing a major or minor preference for each antibiotic class (Dreier and Ruggerone,

2015). Their overexpression has been described as a common intrinsic resistance mechanism, as result of the convergent evolution and adaptation during chronic infections development. This phenomenon represents even the confirmation of the hypothesis formulated by Webber and Piddock (2003), regarding the ancestral role of these pumps and their subsequent adaptation to antibiotics extrusion. Moreover, their involvement in the quorum sensing communication, as well as in the biofilm formation and development, has been demonstrated (Moore et al., 2014).

The two best known and characterized *P. aeruginosa* efflux pumps are MexAB-OprM and MexXY-OprM that have been reported to contribute to the antibiotic treatment failure during *P. aeruginosa* infections (Llanes et al., 2004).

1.2 MexAB-OprM efflux pump

The MexAB-OprM system is formed by the RND transporter MexB, the porin OprM and the adaptor protein MexA. It is the most studied *P. aeruginosa* efflux pump and has been characterized as a model to unravel the exact efflux mechanism and to study its inhibition (Sennhauser et al., 2009). It represents a constitutional component of the *P. aeruginosa* cell and all its proteins are encoded by the bacterial chromosome (Stover et al., 2000); however, it is generally expressed at a basic level, whereas it is overexpressed to mediate the antibiotic resistance (Pan et al., 2016).

The *mexAB-oprM* operon is mainly regulated by the *mexR* gene (Poole, 2005 A). This codes for a repressor that limits the operon expression to basic levels and belongs to the same protein family of the AcrR and MarR proteins, decribed in *E. coli*.

The mutations targeting this gene, that are known to be accumulated as an adaptive mechanism in chronic infections, are responsible for the efflux pump

gene up-regulation and for the extrusion of various antibiotic molecules from the bacterial cells (Srikumar et al., 2000). Further studies have evidenced that the MexAB-OprM efflux pump is, indeed, subjected to other regulators: it is repressed by the second regulator NalD that is dissociated from the operon promoter by the binding with novobiocin (Chen et al., 2016), activating the efflux pump expression; it is activated, in the absence of a functional MexR repressor, by the CprX protein, regulating the cell envelope response (Tian et al., 2016). Both these mechanisms underline that redundant and complex patterns contribute to the expression of this intrinsic resistance mechanism.

The MexAB-OprM pump is involved in the resistance to many classes of antibiotics, specifically fluoroquinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin and several β-lactams, except for imipenem (Masuda et al., 2000).

In the specific case of CF, different studies have reported its involvement in the resistance to cephalosporines, in absence of metallo-β-lactamases (Aghazadeh et al., 2014) and to the ticarcillin derivative temocillin (Buyck et al., 2012) that has proved to be unaffected by a wide range of β-lactamases, including AmpC, and to be a valid alternative to carbapenems in infections sustained by Gram-negative bacteria.

This pump has been described also as particularly important in the ciprofloxacin resistance (Adabi et al., 2015) and it has been reported that, at antibiotic concentrations near to MIC, efflux mutants are first selected in comparison to target mutations. Indeed, fluoroquinolones are an antibiotic class widely used in infections treatment against a wide range of pathogens and constitute a first-line treatment in CF pulmonary infections. The identification of efflux pumps as main resistance mechanism can lead to the development of efficient anti-efflux therapies.

1.3 MexXY-OprM efflux pump

The MexXY-OprM efflux pump is considered the main resistance determinant against aminoglycosides in *P. aeruginosa* (Morita et al., 2012), and it is involved in the "adaptive resistance" to this class of antibiotics (Hocquet et al., 2003). As MexAB-OprM, even this pump is formed by three components, but it presents as peculiarity an operon coding only for the MexX and MexY proteins; the outer membrane component is usually constituted by OprM, but in the clinical isolate *P. aeruginosa* PA7 it is substituted by OprA, indicating that the *mexxy* module can associate with both the porins forming a functional pump. The analysis of this protein complex has even allowed the identification of the periplasmic cleft binding the antibiotics, and it seems to be particularly adapted to bind aminoglycosides (Lau et al., 2014). This efflux pump is, indeed, the only one reported in *P. aeruginosa* as able to extrude this class of antibiotics (Masuda et al., 2000); moreover, it contributes to the MDR phenotype since involved in the resistance to other antimicrobial classes: its overexpression has been linked to the lack of susceptibility to fluoroquinolones, cefepime (Muller et al., 2011), carbapenems (Shen and Fang, 2015) and polymixins (Skiada et al., 2011).

In the aminoglycosides and polymixins case, this efflux pump is the main mechanism of the adaptive resistance. This kind of resistance is not always phenotipically expressed by the pathogen but it is present after induction by substrate antibiotics (Motta et al., 2015).

Moreover, different kind of environmental stimuli, more or less connected with the antibiotic presence, have been reported to induce the *mexxy* operon; these include oxidative stress (Fraud and Poole, 2011), drug-ribosome interactions (Jeannot et al., 2005), translation impairment (Caughlan et al., 2009) and mutations in signal transducing patterns (Muller et al., 2011). However, the main cause of the operon overexpression is the mutation of its repressor, coded by the *mexZ* gene. This is reported as typical in chronic infection isolates and it

is interpreted as a convergent evolution, selecting for the most adapted phenotypes, able to resist to adverse conditions and continuous antibiotic treatment (Marving et al., 2015). These data were confirmed by the analysis of *P. aeruginosa* strains isolated from distinct CF patiens, i.e. children with new infections, chronic infected children and chronic infected adults (Prickett et al., 2017): the *mexZ* mutations resulted more frequent in the last group compared to the others.

The involvement of the MexXY-OprM in aminoglycoside resistance in CF has been evidenced by several authors, underlining both its contribute to a moderate resistance to this antibiotic class (Vogne et al., 2004) and its role in the gradual increase, over time, of aminolycosides MIC values, especially amikacyn and tobramycin (Islam et al., 2009). It partecipates, furthermore, to the classic efflux unbalance characterizing CF *P. aeruginosa* pulmonary infections (Vettoretti et al., 2009) and, thus, represents a putative target to develop a therapy able to counteract MDR *P. aeruginosa* phenotypes.

1.4 Efflux Pumps Inhibitors (EPIs)

The evidence of the efflux pumps role in *P. aeruginosa* antibiotic resistance has led the researchers to focus their attention on these protein complexes, in order to identify possible inhibitors to be adopted in combination with antibiotics and to overcome the MDR phenotype of this pathogen. Over years, different molecules have been reported to potentiate the antibiotic activity by inhibiting its efflux.

The most famous is the phenylalanine-arginine-β-naphthylamide (PAβN), also known as MC207,110, described in 1999 by Renau et al. and further characterized by Lomovskaja et al. in 2001. This compound was reported to mainly inhibit the MexAB-OprM efflux pump, potentiating the activity of antibiotics which are known as substrates of the pump; it showed a particular

synergistic effect with fluoroquinolones, especially with levofloxacin (Sonnet et al. 2012). Moreover, the activity of additional efflux pumps targeting this antibiotic class seemed to be inhibited by this compound. The interaction with the efflux pump was further proved by the lack of modification of the proton gradient across the inner membrane.

However, the same compound was described as an enhancer of the outer membrane permeabilization, increasing the antibiotic activity against Gram-negative bacteria (Lamers et al., 2013).

Another compound showing efflux pumps inhibition is the carbonyl cyanide 3-chlorophenylhydrazone (CCCP); however, it cannot be considered a true EPI, since it does not directly bind the components of the efflux systems, but it acts by decreasing the proton gradient across the bacterial inner membrane, thus depleting the motive force responsible of efflux (Nelson, 2002). Indeed, it has been reported as effective not only against *P. aeruginosa* (Adabi et al., 2015), but even against *Acinetobacter baumannii* (Park and Ko, 2015) and *E. coli* (Paltansing et al., 2013), and it is often used in phenotypical assays to demonstrate the efflux contribution to antimicrobial resistance. However, its effect is shown mainly in combination with fluoroquinolones, as the PAβN.

Finally, the extract of different plants containing berberine has demonstrated to have efflux inhibiting activity against the MexXY-OprM efflux pump (Morita et al., 2016), increasing *P. aeruginosa* susceptibility to aminoglycosides.

This compound was already known for its antimicrobial activity, especially in enterococcal infections (Ball et al., 2006) and has now proved to be effective even against Gram-negative bacteria.

A common problem shared by all these compounds is their toxicity, a feature that limits their use in possible combination therapies with other antimicrobials (Askoura et al., 2011). Thus, researchers are focusing their attention on the

identification of novel putative EPIs, showing more efficiency and less toxicity for the development of anti-efflux therapies.

1.5 Aims of the study

This study investigated the role of the MexAB-OprM (Mangiaterra et al., 2017) and MexXY-OprM efflux pumps as main intrinsic resistance determinants in *P. aeruginosa* and allowed the identification of novel putative natural EPIs, to be used in association with known antibiotics, in order to develop new treatment protocols and to counteract the emergence of MDR and XDR *P. aeruginosa* strains. The first task was achieved by characterizing *P. aeruginos*a resistance to different classes of antibiotics known to be efflux pumps substrates and by confirming the constitutional presence of the two efflux pumps in *P. aeruginosa* genome.

The second goal was obtained by testing different combinations of natural products, identified by virtual screening, in collaboration with the Biomolecular Modeling Group of Dr. Galeazzi, and antibiotics expelled by the two efflux pumps, searching for any improvement or modification of their antimicrobial activities. The toxicity of the tested compounds was then evaluated, in order to select only natural compounds that can be used against MDR *P. aeruginosa* strains in combination therapies, avoiding adverse effects on human body.

2. MATERIALS AND METHODS

2.1 Bacterial strains

The involvement of efflux pumps in *P. aeruginosa* antibiotic resistance was assessed characterizing 25 CF isolates, recovered from the Microbiology Laboratory of AO "Ospedali Riuniti di Ancona" (Italy). Strains were selected according to their resistance to antibiotics known as MexAB-OprM substrates (i.e. ciprofloxacin, meropenem, piperacillin and ceftazidime) and to tobramycin, substrate of MexXY-OprM, that was assessed by routine antimicrobial susceptibility tests in the microbiology laboratory. This collection included the strain *P. aeruginosa* C24, used for the induction of VBNC forms in *P. aeruginosa* biofilms (Chapter 2, Material and methods, Bacterial strains). All strains were plated in MH agar, subcoltured in MC agar and stored as MH broth-cultures, supplemented with 20% glycerol, at -80°C.

The *P. aeruginosa* strains PAO1 and PA14, used in phenotypic and genotypic characterization assays, were kindly provided by Olivier Jousson, Integrated Biology Center, University of Trento (Italy), whereas the *P. aeruginosa* ATCC 27853 belongs to the Microbiology laboratory (DiSVA) strain collection.

2.2 Chemicals

All antibiotics used were purchased from Sigma-Aldrich.

The tested natural products were selected by an *in silico* high-throughput virtual screening, performed by the Biomolecular Modeling Group on the SPECnet ZINC database (about 1500 compounds). Informations about the compounds activities were retrieved from the ZINC and associated Database (www.zinc.org). Experiments were first performed on the MexAB-OprM efflux pump, targeting the inner membrane transporter MexB. Briefly, the inhibitors binding sites on the protein were identified by mapping on it the bindising sites

of the PAβN on the AcrB protein of *E. coli*, homologue of MexB. Then molecular docking experiments were performed using the software AutoDock Vina 1.1.2 to screen the selected database for molecules showing a good binding to the recovered sites on MexB; the best scores were tested again using the software AutoDock 4.2.1 to refine the recovered poses and the relative energies.

The complexed MexB-inhibitor models were then assessed for molecular dynamics using the OPM server, mimicking the physiological conditions of the efflux pump in the bacterial cell.

The top ten hits showing nanomolar activities were then selected and purchased from SPECs (www. specs.net), to be tested in *in vitro* microbiological assays.

The same approach was applied in the identification of MexXY-OprM inhibitors, targeting the MexY transporter. Since the protein 3D model is not available, it was built by homology modeling, using the SWISSMODEL server, and confirmed using the I-TASSER server. As preliminary data, its amino acid sequence, retrieved from the SWISSPROT database, and the 3D-structural coordinates of AcrB and MexB, retieved from the related PDB models, were used.

The selected compounds were prepeared at the concentration of 10 mg/ml and stored at -20°C.

2.3 *In vitro* microbiological tests

2.3.1 Phenotypic and genotypic characterization of *P. aeruginosa* strains

Antibiotic susceptibility tests. The 5 antibiotics MIC was determined according to the agar dilution method, following the CLSI guidelines (see Chapter three, Material and methods, MIC determination). *P. aeruginosa* ATCC 27853 was used as reference strain.

PCR detection of resistance genes. The carriage of the *mexB* and *mexY* genes, coding for the inner transponters of MexAB-OprM and MexXY-OprM respectively, was sought by PCR assays, using the protocols described by Oh et al. (2003) and *P. aeruginosa* DNA extracted as crude cell lysates (Hynes et al., 1992). *P. aeruginosa* PAO1 DNA was used as positive control. Regarding tobramycin resistance, PCR assays targeting the *ndvB* (Beaudoin et al., 2012), *rmtA* (Yamane et al., 2004) and *ant(2")-Ia* (Michalska et al., 2014) genes were performed as previously described; *P. aeruginosa* PA14 DNA was used as positive control in *ndvB* PCRs.

Efflux activity determination. Efflux activity was assessed by the Cartwheel method, an ethidium bromide accumulation agar test described by Martins et al. (2013). Briefly, overnight bacterial cultures in Tryptic Soy (TS) broth were diluted to an OD_{650}=0.1, then swabbed on TS agar plates with/without different concentrations (from 0.25 to 2.5 µg/ml) of ethidium bromide. After 24h incubation, fluorescence emission was determined. *P. aeruginosa* PAO1, known to negatively regulate *mexY* and to express it at basal level after antibiotic induction, was used as reference strain.

Gene expression assays. The expression of *mexY* was compared in presence and absence of subinhibitory antibiotic concentrations (i.e. 1/4x MIC). Briefly, exponential phase bacterial cultures were diluted to an OD_{625}=0.1 in MH broth (final volume, 8 ml), concentrated in 500 µl and supplemented with 1 ml of

RNAprotect Bacterial Reagent (Qiagen). Bacterial cells were lysed by 3 cycles of mechanical (30s vortexing with acid washed glass beads) and temperature (30s incubation in ice) stress and total RNA extracted by the RNeasy Mini Kit (Qiagen), following the manufacturer instructions. The RNA was then reverse transcribed, using the Quantitech Reverse Transcription Kit (Qiagen), and *mexY* cDNA amplified, with the same protocol described above, using the Rotor-Gene SYBR Green PCR master mix (Qiagen) in a Qiagen Rotor-Gene Q termocycler. Expression data were analysed using the Qiagen's Rotor-Gene Q MDx software (Comparative quantitation programme).

2.3.2 Synergy tests

2.3.2.1 Checkerboard assays

Checkerboard assays were performed as previously described (Isenberg, 1992), using 2-fold scalar dilutions of antibiotics, ranging from 1/64x to 16x MIC, and tested compounds, ranging from 320 to 5 µg/ml. The latter range was selected considering that some of the tested compounds can be dissolved in DiMethyl SulfOxide (DMSO): considering the stock concentration (i.e. 10 mg/ml) the highest limit of the range presents 3% of DMSO in the working solution. Although it exceeds the 1% concentration recommended in CLSI guidelines (2017), it was demonstrated that DMSO concentrations till 10% can be tolerated by some cell lines (Da Violante et al., 2002), including hematopoietic cells (Branch et al., 1994); moreover, a 5-10% DMSO freezing has been indicated as best condition for hematopoietic cell recovery (Kadekar et al., 2016).

In the case of ciprofloxacin and tobramycin, a general range of concentrations from 0.125 to 128 µg/ml was adopted.

Since the tested compound did not show any antibacterial activity, the assay results could not be interpreted by FIC index determination. Synergy was then

evaluated on the bases of the ability of natural compounds to decrease the MIC of the antibiotic. A 3–4 fold MIC decrease was considered a synergistic effect.

2.3.2.2 Killing curves

Time-killing curve analyses were performed as described by Isenberg (1992). Antibiotic concentrations ranging from ¼x to 32xMIC were used alone and in combination with the compounds concentrations resulted active in checkerboard assays against a *P. aeruginosa* bacterial inoculum, standardized at 10^5 CFU/ml. The same inoculum was grown free of antibiotics or compounds as control. The dynamic of the bactericidal effect of the antibiotic-natural compound combination was evaluated by CFUs counts at 2, 4, 6, 8 and 24 hours. The concentration of antibiotic or the combination that induced a decrease of 3log compared to the growth control was defined as bactericidal. As for the checkerboard assay, the results could not be evaluated according to CLSI standards for drugs combinations, and any increase of the bactericidal power of the association compared to that of the antibiotic alone, was considered as synergy.

2.3.2.3 Ethidium bromide accumulation assays

Ethidium bromide accumulation assays were performed in presence/absence of the compounds at the concentrations resulted most effective by checkerboard assays, as previously described by Aparna et al. (2014). Briefly, an overnight *P. aeruginosa* culture was diluted 1:50 in MH broth and incubated at 37°C for 4 h with shaking. After centrifugation at 10000xg for 10 min, the pellet of each culture was washed in PBS and resuspended until to an OD_{600} of 0.1. One-hundred seventy µl of the standardized cultures (final cell concentration $1.7x10^6$ cell/ml), 20 µl of 10 mg/ml ethidium bromide (final concentration 2.5 µM) and 10 µl of the natural compounds were then added to each well of a flat bottomed, black 96-well microtiter plate. PBS was used as blank. For the MexXY-OprM inhibition, the *P. aeruginosa* culture was tested with/without efflux induction by subinhibitory tobramycin concentrations (i.e. 1/4x MIC). The plate was incubated at 37°C and the kinetic of the intracellular accumulation of ethidium bromide was evaluated immediately and after 24 hours-incubation by reading at an excitation of 530 nm and at an emission of 590 nm wavelengths for 30 min at 5 min intervals, using the Synergy HT MicroPlate Reader Spectrophotometer (BioTek). Each condition was tested in triplicate. In the case of MexXY-OprM, an inducible pump, readings were repeated even at 30 min after exposure to the tested compounds. The percentage increase in fluorescence, indicating the ability of the compounds to accumulate ethidium bromide as a consequence of the efflux pump inhibition, was calculated as follows:

$$[(Fta-Ft0)/Ft0 \times 100]$$

Where Fta is the fluorescence emitted at times 5, 10, 15, 20, 25 and 30 min and Ft0 the fluorescence at time 0 min.

2.4 Toxicological analysis-Hemolysis assays

The hemolytic activity of the selected compounds was tested as described by Chongsiriwatana et al. (2008). Briefly, 4 ml of freshly drawn, heparanized human blood were diluted with 25 ml of PBS, pH 7.4. After three washings in 25 ml PBS by spinning at 1000xg for 10 minutes the red blood cells, the pellet was resuspended in PBS to ~ 20 vol%. One-hundred µl of erythrocyte suspension was added to 100 µl of the putative EPIs (1:2 serially diluted in PBS) in a 96-wells flat bottom microtiter plate. The negative and the positive control were 100 µl of PBS and 100 µl of 0.2 vol% Triton X-100, respectively. Each sample was tested in triplicate. After 1 h incubation at 37°C each well was supplemented with 150 µl of PBS and the plate centrifuged at 1200xg for 15 min. The supernatant (150 µl) was transferred in a new plate and its OD_{350} measured using the Synergy HT MicroPlate Reader Spectrophotometer (BioTek). The hemolysis percentage (%) was determined as follows:

$$[(A - A0)/(Atotal - A0)] \times 100$$

where A is the absorbance of the test well, A0 the absorbance of the negative control, and Atotal the absorbance of the positive control; the mean value of the three replicates of each sample was recorded.

3. RESULTS AND DISCUSSION

3.1 MexAB-OprM inhibitors

3.1.1 Bacterial strains characterization

The 25 selected *P. aeruginosa* strains were analysed for their antibiotic resistance and the carriage of the MexAB-OprM efflux pump. All strains were shown to carry the *mexB* gene, coding for the inner transporter, whereas the MIC determination revealed different antibiotics susceptibility levels, as shown in Tab. 5.

Tab. 5. MIC determination of the 25 CF *P. aeruginosa* strains against five efflux pump substrate-antibiotics

	MIC (µg/ml)				
STRAIN	**MEROPENEM**	**PIPERACILLIN**	**CEFTAZIDIME**	**CIPROFLOXACIN**	**TOBRAMYCIN**
C6	**2 S**	**8 S**	**2 S**	**2 R**	**8 R**
C8	**2 S**	**16 S**	**2 S**	**2 R**	**4S**
C15	**>16 R**	**2 S**	**>32 R**	**2 R**	**16 R**
C17	**4 I**	**2 S**	**4 S**	**4 R**	**0.25 S**
C24	**>16 R**	**>64 R**	**>32 R**	**4 R**	**32 R**
C25	**2 S**	**32R**	**32 R**	**4 R**	**8 R**
C26	**16 R**	**64 R**	**>32 R**	**16 R**	**>32 R**
C30	**>16 R**	**4 S**	**>32 R**	**2 R**	**16 R**
C31	**>16 R**	**4 S**	**>32 R**	**1 I**	**16 R**
C33	**1 S**	**8 S**	**2 S**	**1 I**	**2 S**
C34	**1 S**	**8 S**	**4 S**	**2 R**	**0.25 S**
C40	**8 R**	**32 R**	**8 S**	**16 R**	**4 S**
C47	**2 S**	**8 S**	**>32 R**	**16 R**	**2 S**
C49	**0,25 S**	**32 R**	**16 R**	**16 R**	**2 S**
C50	**>16 R**	**>64 R**	**32 R**	**4 R**	**8 R**
AR1	**0,5 S**	**8 S**	**4 S**	**1 I**	**2 S**
AR2	**4 I**	**4 S**	**16 R**	**16 R**	**1 S**
AR5	**0,5 S**	**4 S**	**8 S**	**4 R**	**16 R**
AR10	**4 I**	**8 S**	**2 S**	**2 R**	**0.5 S**
AR11	**0,5 S**	**32 R**	**8 S**	**8 R**	**2 S**
AR12	**4 I**	**64 R**	**8 S**	**4 R**	**0.5 S**
AR13	**8 I**	**>64 R**	**16 R**	**1 I**	**2 S**
AR16	**>16 R**	**>64 R**	**4 S**	**4 R**	**0.5 S**
AR17	**>16 R**	**64 R**	**>32 R**	**8 R**	**8 R**
AR20	**1 S**	**16 S**	**4 S**	**2 R**	**0.5 S**

MICs of the *P. aeruginosa* strains and associated resistance phenotype to five efflux pumps substrates antibiotics. S= Susceptible, I=intermediate, R= resistant.

In particular, the strain *P. aeruginosa* C24 reported the following MIC values (µg/ml), all indicating a resistant phenotype: ciprofloxacin 4, meropenem >16, ceftazidime >32, piperacillin >64, tobramycin 32.

This different susceptibility level showed against the five tested antibiotics suggests a different antibiotic-affinity of MexAB-OprM in agreement with the

hypothesis of Webber and Piddock (2003), about the adaptation of the efflux pumps from an ancestral physiological role to the antibiotic extrusion.

The high levels of MICs observed for some antibiotic does not agree with the low-level resistance usually determined by antibiotic efflux. However, efflux pumps have been demonstrated to be involved in biofilm development, and their expression has been described as potentiated in the biofilm lifestyle; moreover, the mutations occurring in *P. aeruginosa* biofilms, targeting specific hot spots, selects for efflux pumps-overproducing mutants, characterized by a high level resistance to the substrate antibiotics (Soto, 2013; Visaggio et al., 2015).

3.1.2 Chemicals

The *in silico* high-throughput virtual screening, performed on the MexB protein, identified two lead compounds, to be tested in association with antibiotics, shown in Fig 19: I) pregnan-20-one derivative (ZINC08382438) and II) morelloflavone (ZINC26187321).

Fig 19. Models of the three putative MexAB-OprM EPIs, i.e. pregnan-20-one derivative (A) and morelloflavone (B).

The 2 compounds were dissolved in DMSO and H_2O, respectively, and then used in checkerboard assays in the range of concentrations 5-320 µg/ml.

3.1.3 Evaluation of the synergistic activity of the natural compounds selected by *in silico* screening

3.1.3.1 Checkerboard assays

The synergistic activity of the tested natural compounds in association with ciprofloxacin was evaluated by checkerboard assays testing a range of antibiotic concentrations from 128 to 0.125 μg/ml. Both pregnan-20-one derivative and morelloflavone decreased the ciprofloxacin MIC when used at a concentration of 40 μg/ml, causing a 4-8 fold MIC reduction in most *P. aeruginosa* strains, especially in *P. aeruginosa* C24, that reported a MIC decrement from 4 to 0.25 μg/ml (Mangiaterra et al., 2017), as reported in Tab 6.

Table 6. MIC of ciprofloxacin alone (first column) and in combination with different concentrations of pregna-20-one derivative and morelloflavone (no compound, 5, 10, 20, 40 80, 160, 320 μg/ml) against *P. aeruginosa* C24.

	Ciprofloxacin MIC (μg/ml)							
Compound (μg/ml)	0	5	10	20	40	80	160	320
Pregna-20-one derivative	4	1	0,25-0,5	0,25-0,5	0,25-0,5	0,25-0,5	0,25	0,25
Morelloflavone	4	2	2	0,25	0,25	0,25	0,25	0,25

No synergistic effect was detected in the associations involving ceftazidime, piperacillin, meropenem or tobramycin. Interestingly, some *P. aeruginosa* strains did not show any improvement of ciprofloxacin activity even in combination with the two putative EPIs and were all characterized by pyoverdine production. This siderophore has been reported as signal molecule for the expression of metabolic pathways (Beare et al. 2003; Mueller et al. 2007), virulence factors and extracellular polysaccharides. Thus, it can be hypothesized its involvement in the expression of factors binding and sequestering (or destroying) the tested compounds, thus preventing synergy. The observed discrepancies, both involving pyoverdine action and the lack of

synergy with the other antibiotics, are currently being investigated. These data are however consistent with other studies involving different EPIs, that report synergistic combinations mainly with fluoroquinolones (Lomovskaya et al. 2001; Goli et al. 2016), and comparable synergy results.

P. aeruginosa C24 has been used for further synergy assays and in the investigation of MexXY-OprM inhibition.

3.1.3.2 Time killing curves

The effectiveness of the combinations pregnan-20-one derivative/morelloflavone-ciprofloxacin was further investigated by killing curves analysis to assess their bactericidal effect on *P. aeruginosa* C24. Ciprofloxacin alone exhibited a bactericidal effect at the MIC concentration (i.e. 4 µg/ml) after 6 h of exposure, whereas the effect of 1/2x MIC (i.e. 2 µg/ml) resulted bacteriostatic. The presence of each of the two compounds improved this results, as shown in Fig. 20:

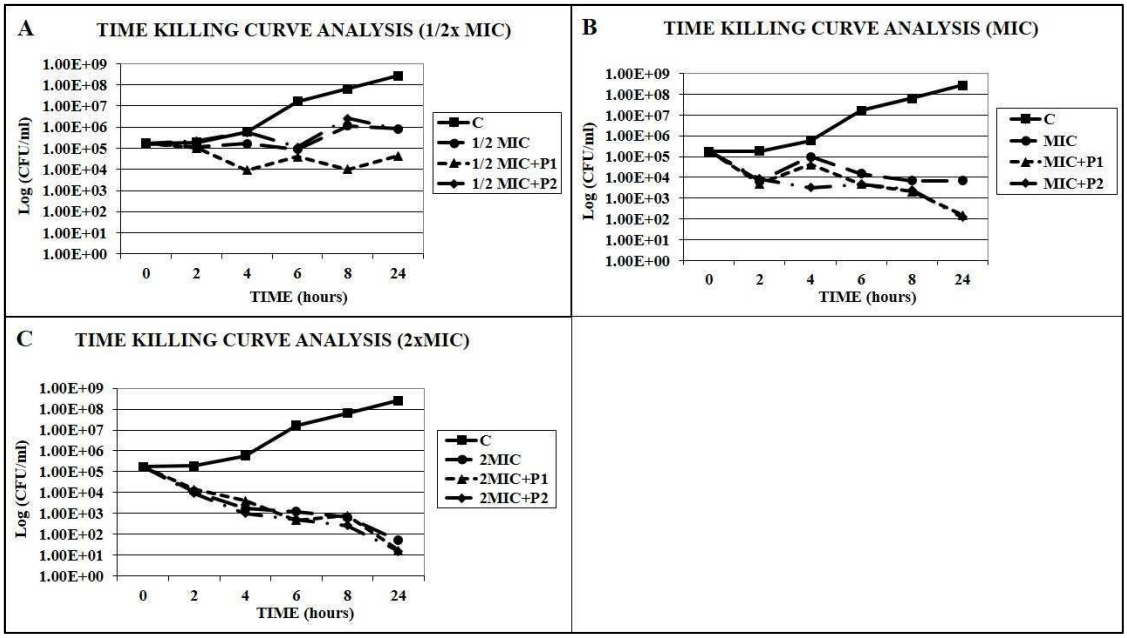

Fig. 20. Time killing curve analysis of *P. aeruginosa* C24 exposed to ciprofloxacin alone and to different combinations of ciprofloxacin (1/2xMIC A, MIC B and 2xMIC C) and 40 µg/ml of pregnan-20-one derivative (P1) or morelloflavone (P2)

The combination pregnan-20-one derivative (40µg/ml)–ciprofloxacin (2µg/ml) produced a 1 log decrease of CFUs counts at 6h of exposure and a 2 log decrease at 24 h. This result was more evident in assays performed with 4 µg/ml (MIC) ciprofloxacin irrespective of which of the two EPIs was used, whereas no significant synergistic effect was detected in combination with 8 µg/ml (2xMIC) antibiotic. The killing curve analysis also revealed different dynamics of the two combinations, the association with pregnan-20-one derivative producing a faster bactericidal effect than the combination with morelloflavone (Fig 20 A and B), although the effects of the 2 compounds at the end (24h) of the experiment were comparable. These data perfectly match with the molecular dynamics prediction that reported a faster stabilization of pregnan-20-one derivative in its binding site whitin the MexB protein and overall comparable results at the end of the simulation. Pregna-20-one derivative and morelloflavone were thus both considered as putative MexAB-OprM EPIs.

3.1.3.3 Ethidium bromide accumulation assays

The morelloflavone and pregnan-20-one derivative activity as MexAB-OprM EPIs was finally confirmed by ethidium bromide accumulation assays, performed using the strain *P. aeruginosa* C24 in absence or presence of the two selected compounds (40 µg/ml). Both compounds showed the ability to decrease ethidium bromide efflux, as indicated by the increase of *P. aeruginosa* C24 fluorescence during the examined 30 min kinetics (Fig. 21). In agreement with the molecular dynamics previsions and the results obtained in time killing assays, the pregnan-20-one derivative exhibited a faster inhibition activity compared to morelloflavone, (i.e. 22%/16% fluorescence increase respectively after 15min and 36%/22.4% after 30min). However, after further 24h exposure to the EPIs, the morelloflavone showed a greater inhibition of the efflux pump than pregnan-20-one derivative (i.e. fluorescence increase of 24.2% and 13%,

respectively). These data suggest that while the pregnan20-one derivative interacts with the MexB protein more directly and in a shorter time, after 30 min morelloflavone causes a more efficient inhibition of the efflux pump, probably as a consequence of its orientation and efficient interaction with the target protein.

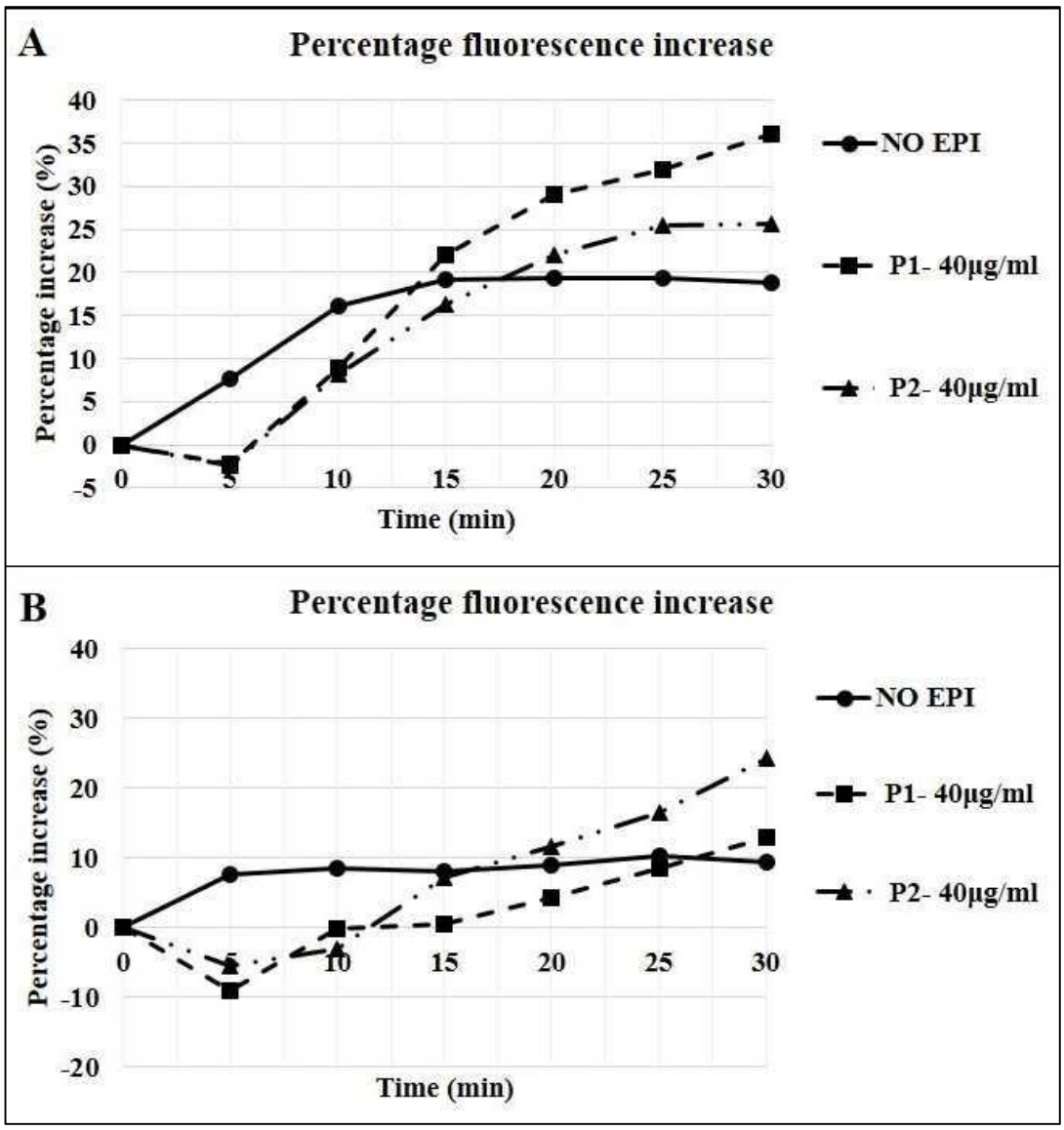

Fig. 21. Kinetic of the *P. aeruginosa* C24 ethidium bromide intracellular accumulation in the presence of Pregnan-20-one derivative (P1) or Morelloflavone (P2) expressed as the percent of fluorescence increase monitored (OD_{590}) immediately (A) and after 24h (B) of exposure to EPIs

3.1.4 Hemolysis assays

The pregnan-20-one derivative and morelloflavone were finally examined in hemolysis assays to exclude any toxic activity, testing a range of concentrations 20-80 μg/ml, including the one resulted active in synergy tests. No hemolytic activity was visibly observed with both compounds at all tested concentrations; however, the pregna-20-one derivative showed an OD_{350} increase of 0,8% (±0.005 and ±0.004, respectively) at 20 and 40μg/ml and of 1.6 % (±0.005) at 80 μg/ml, while morelloflavone showed no hemolytic activity at 20 and 40 μg/ml and a 4 % (± 0.002) increase of haemolysis when tested at 80 μg/ml.

3.2 MexXY-OprM inhibitors

3.2.1 *P. aeruginosa* C24 tobramycin resistance characterization

On the basis of the results obtained with MexAB-OprM inhibitors, *P. aeruginosa* C24 was adopted as model for the study of MexXY-OprM EPIs. This strain was found to carry only the chromosome encoded aminoglycoside resistance determinants *mexY* and *ndvB*, whereas the PCR assays targeting the other aminoglycosides resistance genes gave uniformly negative results.

The efflux efficiency was further characterized, at phenotypical level, through the ethidium bromide accumulation agar test (Cartwheel method), that reported a minor fluorescence, i.e. a greater ethidium bromide efflux from *P. aeruginosa C24* in comparison with the reference strain *P. aeruginosa PAO1* (data not shown). *MexY* gene expression with/without induction with subinhibitory (1/4x MIC) concentrations of tobramycin was evaluated in both *P. aeruginosa C24* and *P. aeruginosa PAO1*; *P. aeruginosa* C24 either induced and not induced exhibited a 5 times higher gene expression compared to *P. aeruginosa* PAO1; no significant difference was noticed between the strain grown in medium unsupplemented/supplemented with antibiotic, indicating the constitutive

overexpression of the efflux pump and the suitability of the selected strain for further analyses.

3.2.2 Putative EPI identification

The MexY homology modeling allowed the identification of 2 further binding sites in comparison to the MexB model, specifically one located in its inner membrane alpha-helices and the other in the oligomerization surface; however, both sites were not considered in the EPIs binding docking, since showing lower protein binding activity than the previous analyzed sites. Among the compounds present in the selected database, only one, i.e. limonin (Fig. 22), showed a binding energy lower than -10.0 kcal/mol (corresponding to nanomolar activity) and a good binding activity for both sites, and thus was selected for *in vitro* microbiological tests.

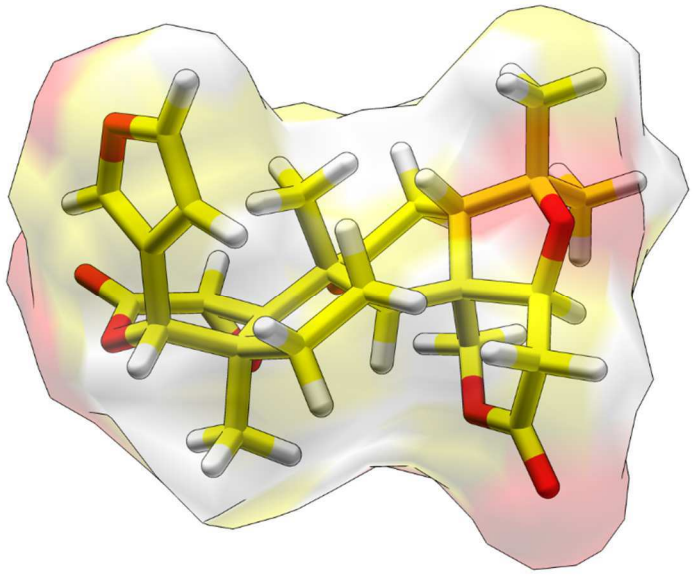

Fig. 22. Chemical structure of limonin, i.e. the putative MexXY-OprM EPI.

Limonin was suspended in DMSO 100% and then used in subsequent analyses in the range of concentrations 320-5 µg/ml.

3.2.3 Synergy tests

3.2.3.1 Checkerboard assays

The synergistic effect of the combination limonin-tobramycin was tested in checkerboard assays, performed on *P. aeruginosa* C24 using tobramycin and limonin doubling concentrations, from 0.125 to 128 µg/ml and from 5 to 320 µg/ml, respectively. It must be noted that the same antibiotic, in association with pregnan-20-one derivative and morelloflavone, did not show any synergy against the investigated strain, indicating that the inhibition of MexAB-OprM is not connected with the restoration of tobramycin susceptibility in *P. aeruginosa*. An evident synergy was detected when using 320 µg/ml of limonin, which determined a 16-fold decrease (from 32 to 2 µg/ml) of tobramycin MIC, corresponding to a shift from the resistant to the susceptible phenotype. This is consistent with the results obtained with the MexAB-OprM selected EPIs in combination with ciprofloxacin; moreover, since no extrachromosomal resistance gene was detected and *ndvB* is poorly expressed in planktonic conditions (Mah et al., 2003), it is reasonable to assume that the MexXY-OprM inhibition, due to the limonin binding, is responsible for the tobramycin MIC decrement. The molecule could thus be considered a putative EPI.

3.2.3.2 Time killing curves

The bactericidal effect of the tested combination was assessed in time killing curves assays, using 320 µg/ml limonin (i.e., the concentration resulted active in checkerboard assays) and tobramycin doubling concentrations from 1/2x to 32xMIC (i.e. from 16 to 1024 µg/ml). A bactericidal effect against *P. aeruginosa* C24 was observed only for tobramycin concentrations corresponding to 16 and 32xMIC (Fig.23). Moreover, the association tobramycin-limonin, while generally produced an improvement of the antibiotic bacteriostatic effect, caused a modest (0.5-1 log) improvement of tobramycin bactericidal activity, after 8 h and 24 h exposure to the combination antibiotic-EPI, respectively. To optimize the limonin effect, further studies are in progeress, focusing on limonin chemical modification and on the use of a carrier able to improve the EPI binding to the MexY protein.

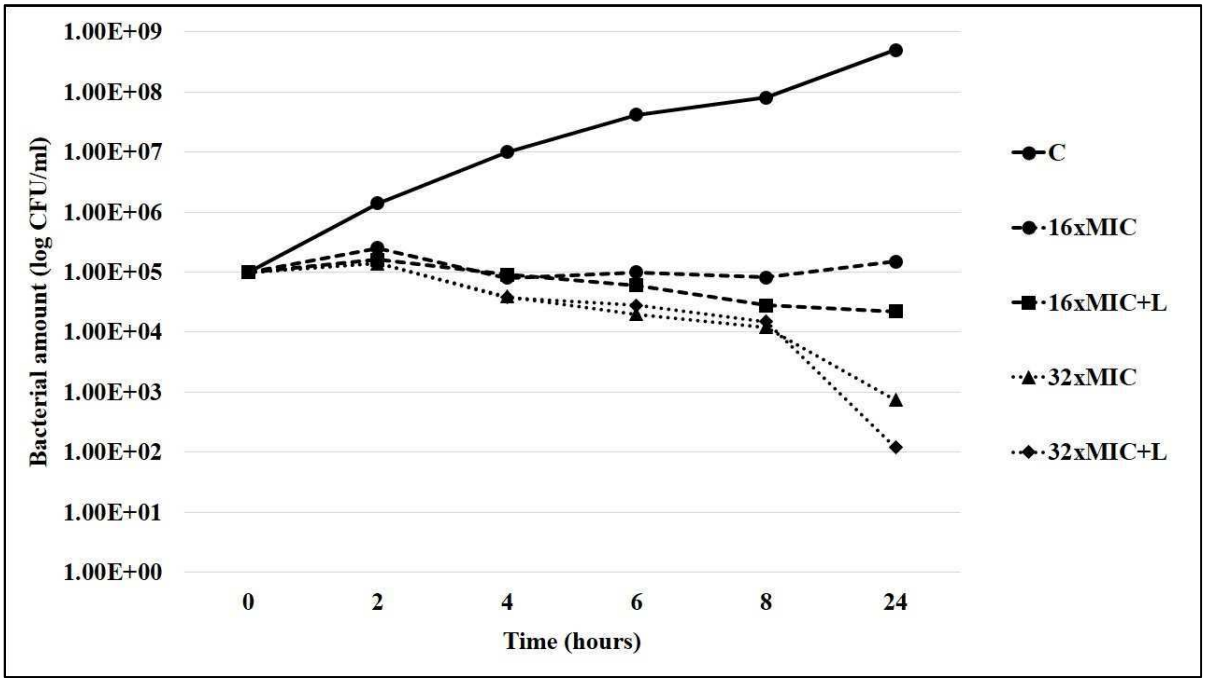

Fig. 23. Time killing curve of *P. aeruginosa* C24 (C) using different tobramycin concentrations, alone or in combination with 320 µg/ml of limonin (+L). 512 (16xMIC) and 1024 µg/ml (32xMIC) of tobramycin were used.

3.2.3.3 Ethidium bromide accumulation assays

The EPI activity of limonin was finally proved through ethidium bromide accumulation assays, testing the same concentration resulted active in checkerboard assays (i.e. 320 µg/ml). Since MexXY-OprM is inducible, *P. aeruginosa* C24 was grown in presence or absence of 1/4xMIC of tobramycin, and further readings after 30 min of limonin exposure were added. The results are shown in Fig. 24. In the first 30 minutes right after the addition of ethidium bromide, no difference was detected between the cultures treated and untreated with limonin; this may be explained considering that the efflux system has to be induced to extrude the dye. After this lapse of time (T0.5), the presence of limonin determined a 15.03% and 20.93% fluorescence increase of induced (i.e. grown with ¼x MIC tobramycin) and not induced (i.e. grown without antibiotic) efflux system, respectively. The same cultures tested in the absence of limonin showed a 7.78% (induced) and 9.23% (not induced) increase of fluorescence (Figure 24A). This result was further confirmed by readings performed after 24 h (T24), reporting a 43.72%/46.07% fluorescence increase for cultures exposed and not-exposed to tobramycin, respectively, in presence of limonin. In absence of the compound, the fluorescence increase of the same cultures was 24.05% and 18.48%. These data further support the inhibitory activity of limonin against the MexXY-OprM efflux system.

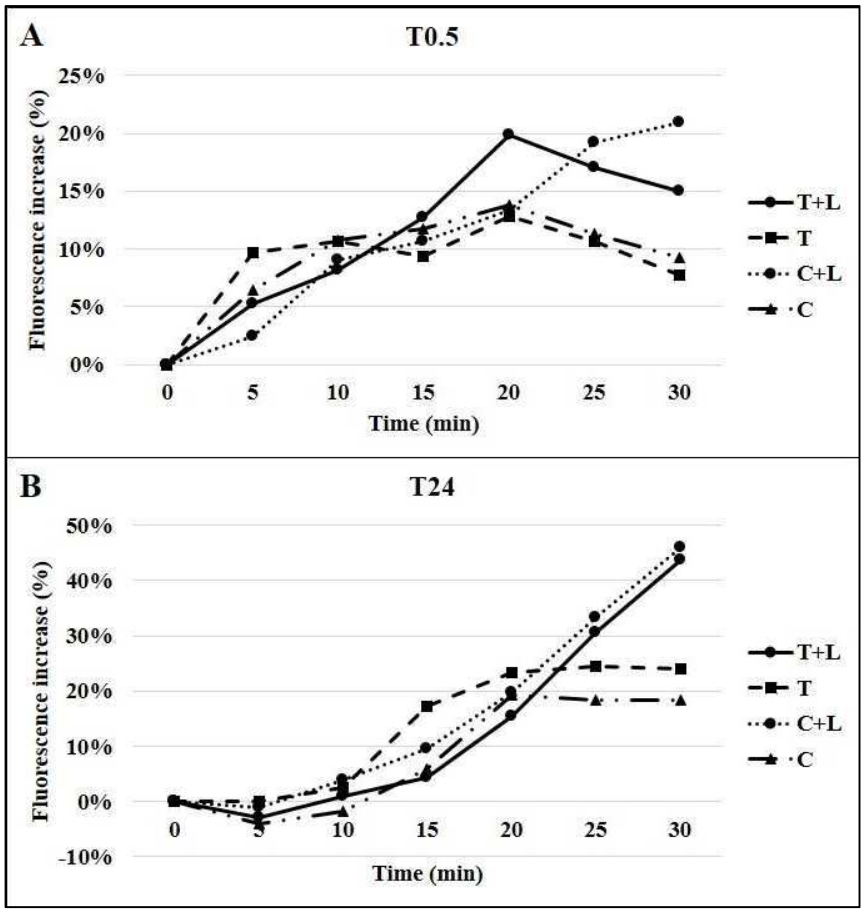

Figure 24. Kinetic of the ethidium bromide accumulation by *P. aeruginosa* C24, (expressed as increase (%) of fluorescence) performed (A) after 30 min (T0.5) and (B) after 24 h (T24) from the addition of ethidium bromide. Bacterial cells were grown in the presence (T) or absence (C) of 1/4xMIC of tobramycin and then tested with (L) or without limonin 320 µg/ml

3.2.4 Hemolysis assays

The possible toxic effect over human cells of the DMSO used to dissolve limonin was excluded by hemolysis assays, performed testing the same limonin concentrations used in checkerboard assays, all suspended in 3% DMSO, i.e. the same percentage present in the limonin concentration (320 µg/ml) synergistic with tobramycin.

Although exceeding the 1% DMSO concentration, none of the tested limonin solutions resulted toxic, including that containing 320 µg/ml of the compound, which showed a value of hemolysis of 2.6%±0.002; the other concentrations reported all hemolysis values <1%. These results show that limonin is not cytotoxic and might be suitable for the development of combination therapies with tobramycin.

4. CONCLUDING REMARKS

The continuous emergence of MDR and XDR *P. aeruginosa* strains represents a great threat to public health, especially in conditions such as CF, where this pathogen develops chronic and persistent infections. The identification of the main mechanisms underneath this antibiotic resistance is a primary goal of microbiological research. Efflux pumps constitute a main, intrinsic resistance determinant, being responsible for the failure of various antibiotic treatments, especially the ones based on ciprofloxacin and tobramycin. The efflux inhibition seems, thus, a valid strategy to counteract the infections sustained by MDR and XDR *P. aeruginosa*. In this study, two putative natural EPIs specific for the MexAB-OprM efflux pump and one specific for MexXY-OprM have been identified and characterized, reporting the decreased of ciprofloxacin (8 fold times) and tobramycin (16 fold times) MIC respectively and, in the former case, a consistent increase (2 log) of the antibiotic bactericidal effect. The inhibitory activity of *P. aeruginosa* efflux exerted by the three compounds was demonstrated by ethidium bromide accumulation assays and their lack of toxicity by hemolysis tests. These molecules seem thus to represent valid lead compounds for the development of combined therapies with known antibiotics; further studies will be dedicated to enhance their EPI activity, in order to better counteract the efflux-mediated resistance in *P. aeruginosa*.

CONCLUSIONS

P. aeruginosa pulmonary infections constitute the main threat for CF patients, due to their recurrent exacerbations and the resulting respiratory failure.

Many features of these infections have been elucidated, including the initial colonization steps, the biofilm formation and its involvement in the infection development, the virulence factors involved, the convergent evolution *P. aeruginosa* strains are subjected to and the resistance mechanisms responsible for the antibiotic treatments failure. However, several features that coud produce a great impact on the clinical treatment and management of *P. aeruginosa* infections remain not investigated and without clear insights. First of all, *P. aeruginosa* infection diagnosis, performed by classical cultural methods, has proved to be unsuitable to reliably determine the lung colonization dynamics of the pathogen: the emergence of atypical, and especially slow-growing, phenotypes hampers the final diagnostic result. Moreover, cultural techniques are unable, due to their low sensibility, to reveal the presence of small populations of persister and VBNC *P. aeruginosa* forms, considered the cause of the pulmonary infection recurrence. The diagnostic protocol presented in this thesis, based on the molecular detection and quantification of this pathogen by targeting a species-specific gene, has resulted not only to be a reliable diagnostic tool, able to implement the routinely adopted protocols, but even to reveal the presence of non culturable *P. aeruginosa* forms, probably connected with the infection recurrence. Till now the importance of the detection of these forms has been understimated and there are only by few reports (Deschaght et al., 2013; Le Gall et al., 2013) dealing with the development of a method able to reliably quantify the pathogen. This can be due to the still unclear features of the dormant/unculturable/persistent bacterial forms and differences and similarities among them. In particular, the study of the VBNC forms, lacking to growth in bacteriological media and presenting unclear features, is problematic.

VBNC induction in biofilm has been observed, but the specific factors involved in their development remain unknown. In this study, subinhibitory tobramycin

concentrations have demonstrated to induce a hormetic modulatory effect on *P. aeruginosa* cells embedded in biofilm, stimulating the development of a stable VBNC population, whose mantainance for over 2 months has been demonstrated.

Although this result is still preliminary, it adds new insights into the bacterial response to the stress condition represented by antibiotic exposure.

Indeed, different studies (Hoffman et al., 2005; Gotoh et al., 2008) have highlighted the positive influence of antibiotics in biofilm formation and it is well known that this lifestyle is characterized by both increased antibiotic resistance and tolerance, consistent with the up-regulation of specific resistance mechanisms. However, the exact role of antibiotics in the modulation of gene expression is still poorly investigated. Antibiotic effects are usually examined at inhibitory concentrations, whereas the world of the subinhibitory concentrations is often neglected; although these are the antibiotic amounts often present within biofilms, especially in the deeper layers, only few studies (Jørgensen et al., 2013; Wassermann et al., 2016) have started to determine and appreciate their effects on bacterial phenotype and further efforts have to be dedicated to unravel the variety of bacterial response to antibiotic treatments.

Bacterial persistence represents another particular riddle, especially regarding its genetic regulation. Since it is generally considered a stochastic event, induced by several stress conditions, including the presence of toxic compounds, it is reasonable to think that its development might be modulated by general genetic pathways. In this thesis, the signal transducing pathway Gac/Rsm has proved not to contribute, at least directly, to persistence formation, since no difference was detected in the *P. aeruginosa* persisters amounts between the presence/absence of the regulator protein RsmA. Although this system is known to influence various *P. aeruginosa* pathways, including the switch from planktonic to sessile lifestyle, it seems like that other regulatory patterns are involved in this

particular stress response. Moreover, the c-di-GMP, a second messenger deeply involved in biofilm control and formation, seems both to be upregulated in absence of RsmA and to characterize a precise response of different *P. aeruginosa* genotypes to antimicrobial pressure. Further studies are in progress to better characterize the role of this molecule, the genetic patterns involved in its synthesis/degradation, and their involvement in the persistent cells formation and maintenance during antibiotic treatment. The different ability to induce persister cells by different antibiotic classes is currently under investigation, as well as their link with the VBNC forms and the genetic pathways involved in the development of these two dormant forms in *P. aeruginosa* biofilms.

Finally, several studies have focused on *P. aeruginosa* pulmonary infections in CF patients and on their antibiotic tolerance and therapy failure as well as on the identification of novel resistance determinants, responsible for the lack of the antibiotic action.

Efflux pumps have been recognized as one of the main resistance determinants in this pathogen, able to exclude different antibiotic classes and preventing several treatment options from being adopted. Their expression is remarkably upregulated in chronic infections, as conseguence of both the accumulation of mutations in their regulatory genes and the biofilm growth. The efflux inhibition has been described as a valid option for the development of novel combined therapies, in order to counteract *P. aeruginosa* antibiotic resistance, especially in MDR and XDR strains. Although a great interest and effort have been dedicated to the identification of *P. aeruginosa* EPIs, the studied compounds often show toxic properties, preventing their use in antibiotic treatment. In this thesis, 3 natural molecules have been identified as putative EPIs, specifically 2 (i.e. pregna-20-one derivative and morelloflavone) for the MexAB-OprM efflux pump, the most studied and characterized one in *P. aeruginosa*, and 1 (i.e. limonin) for the MexXY-OprM, considered the main aminoglycosie resistance determinant in this pathogen. All the three compounds have been shown to

inhibit *P. aeruginosa* efflux and to improve the bacteriostatic action of both ciprofloxacin and tobramycin, the substrates of MeXAB-OprM and MexXY-OprM, respectively; in the former case, a potentiation of ciprofloxacin bactericidal effect was also noticed.

None of the 3 compounds showed cytotoxic activity, thus resulting optimal putative EPIs, able to be safely adopted in association with known antibiotics in combined therapies against *P. aeruginosa*. They therefore represent lead compounds, to be modified and optimized, though chemical modification or the use of carrier molecules, to improve their EPI action and to obtain a greater antibiotic effect against MDR and XDR *P. aeruginosa* strains.

In conclusion, despite the acquired knoweldge about *P. aeruginosa* pulmonary infections in CF and the contribution provided by the results obtained in this thesis, a long list of questions has still to be answered, and microbiological research must focus its attention on these topics to unravel the related unsolved problems. The better characterization of these features will allow to developed specific treatment protocols able to avoid the development of chronic infections and to improve the pathogen clearence from the lung environment, assuring a better quality and expectansy of life to CF patients.

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