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IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* RELEASED PROTEINS: EFFECTS OF OXYGEN LIMITATION AND AZITHROMYCIN TREATMENT IN CLINICAL AND LABORATORY STRAINS

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ABSTRACT

Colonization by *Pseudomonas aeruginosa* (Pa) is a hallmark of lung disease in cystic fibrosis (CF), where microaerobic conditions develop as a consequence of disease progression. Conditioned medium (CM) obtained from Pa clinical strain AA2, unlike the CM from laboratory strain PAO1, induces in airway epithelial cells IL-8 mRNA in both aerobic and microaerobic conditions. The effect was impaired by protease digestion. Shotgun proteomic analysis (Multidimentional protein identification technology: MudPIT) of conditioned medium of PAO1 and AA2 identified 451 and 235 individual proteins.

Various proteins were found differentially regulated between strains and culture conditions. Among these eleven different proteases were found released by the AA2 strain, while fewer peptides of only four of them were detected in the PAO1 strain. Ecotin, a protease inhibitor, was found to be highly represented in PAO1 in comparison with AA2 grown in microaerobiosis. These results were confirmed by functional assay (zymography) and western blotting. The pattern of expression of several proteases and their inhibitor ecotin correlates with pro-inflammatory activity *in vitro* better than other candidate virulence factors. Only 31% of the Pa strains isolates from chronically infected CF patients expressed detectable metalloprotease activity while all the isolates derived from sporadically infected individuals scored positive (individual strains analyzed: 42, $p<0.002$). These results suggest that high-throughput approaches are critical to unravel the complexity of the pro-inflammatory microenvironment associated to the presence of Pa and to facilitate the identification of key molecules involved in Pa biology/pathology.

There is considerable interest in the use of azithromycin (AZM) for the treatment of lung disease in patients with cystic fibrosis. Although its mechanism of action as an inhibitor of bacterial protein synthesis has been well established, it is less clear how AZM ameliorates the lung disease associated with *P. aeruginosa*, which is considered to be resistant to the drug. Modulation of Pa virulence factors was suggested as mechanism for AZM beneficial effects in CF patients. We tested

the effects of azithromycin on clinical isolate AA2 to establish how this drug might interfere with the production of bacterial virulence factors that are relevant to the pathogenesis of airway disease in CF patients. We demonstrated that the increase of IL-8 mRNA in CF epithelial cells induced by CM from AA2 was significantly reduced when the clinical strain was grown in the presence of AZM, suggesting that this macrolide reduces Pa pathogenicity. In the attempt to gain information on the identity of the molecules released by Pa clinical strain before and after treatment with AZM we applied MudPIT. We found 5 upregulated and 7 downregulated proteins in CM from AA2 incubated with AZM. Peptides from the alkaline metalloproteinase precursor (APR) were less represented in CM derived from AA2 strain grown in presence of AZM than in those from the same strain cultured in absence of this macrolide. AZM was observed also to decrease the metalloprotease activity and APR expression in CM of Pa isolates derived from sporadically infected individuals while any effect was detected in CM of Pa isolates from chronically infected CF patients. These results was validated by means of zymography assay and western blot technique. The MudPIT analysis of released proteins from Pa clinical isolate grown alone and in presence of AZM gives suggestion on the macrolide ability to decrease the expression of substances that contributes to Pa virulence, such as alkaline metalloproteinase. The effects of AZM on the expression and release of selected polypeptides by Pa strains may help to explain the clinical benefits associated with macrolide therapy.

INTRODUCTION

Cystic fibrosis

Cystic fibrosis (CF) is the most common lethal monogenic disorder in populations of northern European descent, among whom the disease occurs in approximately 1 in 3000 births. Birth prevalence varies from country to country, and with ethnic background. For example, the disease occurs in roughly 1 in 3000 white Americans, 1 in 4000–10000 Latin Americans, and 1 in 15000–20,000 African Americans [1]. Cystic fibrosis is uncommon in Africa and Asia, with a reported frequency of 1 in 350000 in Japan [2].

CF is caused by dysfunction of a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is expressed in many epithelial cells and blood cells. CFTR belongs to a family of transmembrane proteins called adenosine triphosphate (ATP) binding cassette transporters, and functions mainly as a chloride channel in the apical membrane of epithelial cells lining the target organs (lung, intestine and sweat gland). It has many other regulatory roles, including inhibition of sodium transport through the epithelial sodium channel, regulation of the outwardly rectifying chloride channel, regulation of ATP channels, regulation of intracellular vesicle transport, acidification of intracellular organelles, and inhibition of endogenous calcium-activated chloride channels [3-7]. CFTR is also involved in bicarbonate–chloride exchange. A deficiency in bicarbonate secretion leads to poor solubility and aggregation of luminal mucins [8].

The *CFTR* gene encompasses approximately 180000 base pairs on the long arm of chromosome 7. The protein contains 1480 amino acids. More than 1500 disease-associated mutations have been described in the coding sequence, messenger RNA splice signals, and other regions. It is important to understand that the functional consequences of many of these mutations are poorly understood and the majority of these mutations are rare (The Cystic Fibrosis Mutation Database. http://www.genet.sickkids.on.ca/cftr. *Accessed March 20,* 2009). These mutations can be classified on the basis of the mechanism by which they are believed to cause disease (Fig. 1) [9].

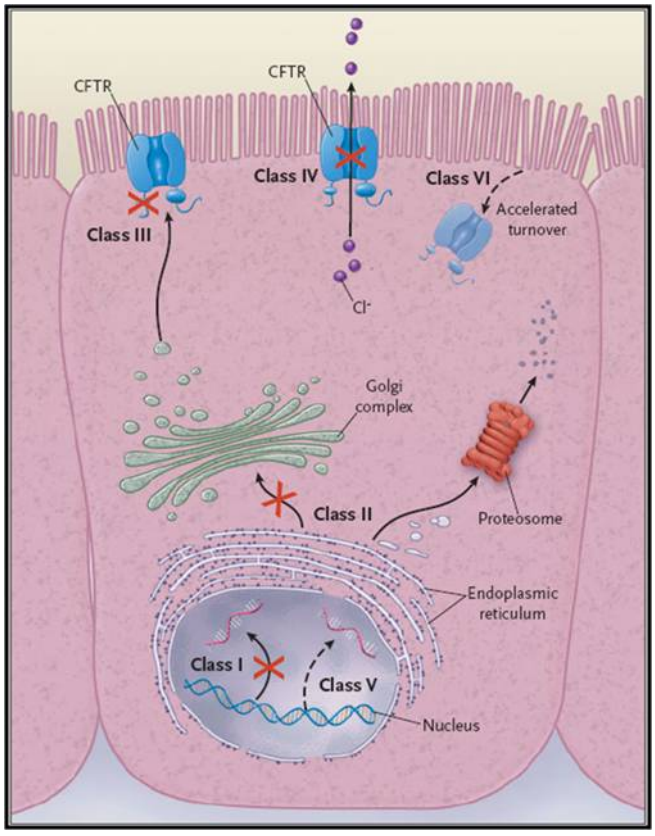


Figure 1. Categories of *CFTR* Mutations. Classes of defects in the *CFTR* gene include the absence of synthesis (class I); defective protein maturation and premature degradation (class II); disordered regulation, such as diminished ATP binding and hydrolysis (class III); defective chloride conductance or channel gating (class IV); a reduced number of CFTR transcripts due to a promoter or splicing abnormality (class V); and accelerated turnover from the cell surface (classVI). (From Reference 9)

The most common mutation, which is termed ΔF508 and is present in approximately 70 percent of defective *CFTR* alleles and in 90 percent of patients with cystic fibrosis in the United States, is categorized as a class II defect. CFTR with the ΔF508 mutation lacks a phenylalanine (F) residue at position 508. The defective protein retains substantial chloride-channel function in cell-free lipid membranes. When synthesized by the normal cellular machinery, however, the protein is rapidly recognized as misfolded and is degraded shortly after synthesis, before it can reach its crucial site of action at the cell surface. Like ΔF508, several other clinically important mutations — such as N1303K, G85E, and G91R — lead to misfolded CFTR protein that is prematurely degraded. About 5 to 10 percent of *CFTR* mutations are due to premature truncation or nonsense alleles (designated by "X," such as G542X, a class I mutation). Other *CFTR* mutations encode properly processed, full-length CFTR protein that lacks normal ion-channel activity. For example, the G551D mutation

(class III) is believed to possess little or no chloride-channel function *in vivo* because of abnormal function of a nucleotide-binding domain, resulting in disordered regulation. The A455E mutation (class IV) exhibits only partial CFTR ion-channel activity, a feature that probably explains a less severe pulmonary phenotype [10]. Other mutation classes include reduced numbers of CFTR transcripts (class V) and defective CFTR stability at the cell surface (class VI).

CF Pathophysiology: pulmonary infection and inflammation

Cystic fibrosis is a complex disease affecting a number of organ systems including the lung and upper respiratory tract, the gastrointestinal tract, pancreas, liver, sweat glands and the genitourinary tract. Although CF is a multisystem disease, lung involvement is the major cause of morbidity and mortality. In CF, a failure of lung defense leads to the establishment of bacterial endobronchitis accompanied by intense inflammation and airway destruction. In the human lung, thick, tenacious secretions obstruct the distal airways and submucosal glands, which express CFTR [11]. Ductular dilatation of these glands (associated with blockage by mucus) and the plastering of airway surfaces by thick, viscous, neutrophil-dominated mucopurulent debris are among the pathological hallmarks of the disease. Pathogens such as *Pseudomonas aeruginosa* (Pa), *Burkholderia cepacia, Staphylococcus aureus,* and *Haemophilus influenzae* become well established within firmly fixed airway secretions in patients with cystic fibrosis and are not effectively eradicated.

There are several hypotheses regarding how CFTR dysfunction leads to development of these infections. Four hypotheses are outlined below; it is possible that aspects of all four contribute to the pathogenesis of the disease.

The low-volume hypothesis (Fig 2, panel D) postulates that the loss of inhibition of epithelial sodium channels, because of CFTR dysfunction, leads to excess sodium and water reabsorption, resulting in dehydration of airway surface materials [12] [13] [14]. Concomitant loss of chloride efflux prevents the epithelium from correcting the low airway surface water volume. The subsequent decrease in periciliary water volume results in a reduction in the lubricating layer between

epithelium and mucus, with compression of cilia by mucus causing inhibition of normal ciliary and cough clearance of mucus. According to this hypothesis, mucus on the epithelium forms plaques with hypoxic niches that can harbour bacteria, particularly *Pseudomonas aeruginosa* [14 15].

The alternative high-salt hypothesis contends that the airway epithelial surface in patients with cystic fibrosis behaves similarly to sweat ducts, in that CFTR is the major pathway for counter-ion absorption (Fig. 2, panel C**)**. This hypothesis argues that in the absence of functional CFTR, excess sodium and chloride are retained in airway surface liquid [16 17]. The increased concentration of chloride in the periciliary layer disrupts the function of important innate salt-sensitive cationic antimicrobial peptides, defensins, (eg, human β-defensin 1), allowing bacteria that are cleared by normal airways to persist in lungs [18]. However, not all defensins are salt-sensitive, it is difficult to prove or disprove that the airway surface liquid in CF is hypertonic, and most studies have found that airway surface liquid is isotonic in CF.

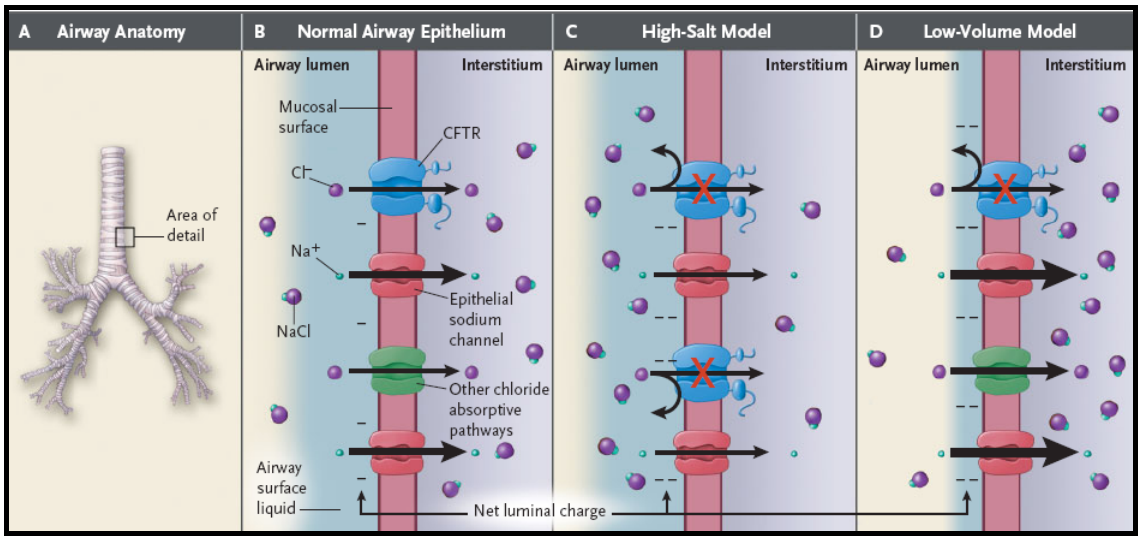


Figure 2. Models Explaining the low-volume hypothesis and high-salt hypothesis. Under normal conditions, sodium chloride is absorbed from the airways (Panel A). The first step of this process uses sodium and chloride absorptive pathways present in the luminal (apical) membranes of airway-surface epithelial cells, designated as the mucosal surface (Panel B). In the high-salt model (Panel C), the situation resembles that of the sweat duct, in which the absence of CFTR leads to the inability to reabsorb chloride ion from airway-surface liquid. In the low-volume model (Panel D), both sodium and chloride are hyperabsorbed. This model predicts a depletion in the volume of airway-surface liquid (shown in blue). The thickness of the arrows corresponds to the degree of movement of ions. (From Reference 9)

The cell-receptor hypothesis suggests that cystic fibrosis cell organelles are more acidic [19] or alkaline [20] than organelles from normal cells, and that altered pH leads to reduced sialysation of glycoconjugates on cystic fibrosis epithelial cell membranes. Increasing numbers of asialoGM1 molecules—a receptor for many bacterial respiratory pathogens—have been reported on cystic fibrosis epithelial cells, resulting in increased binding of *P. aeruginosa* and *S. aureus* on these cells [19] [20]. In normal hosts, *P. aeruginosa* binds to functional CFTR and initiates an innate immune response, which is rapid and self-limiting. In patients with cystic fibrosis, an increase in asialo-GM1 in apical cell membranes allows increased binding of *P. aeruginosa* and *Staphylococcus aureus* to airway epithelium, without initiation of the CFTR-mediated immune response [21]. The result is that in cystic fibrosis, the rapid, self-limiting response that eliminates *P. aeruginosa* from the airways is lost at the same time as there is enhanced attachment of bacteria to the epithelial surface. However, more recent studies revealed that both *P. aeruginosa* and *S. aureus* are mainly located in the mucus layer on respiratory epithelial cells, rather than directly on cell membranes, which makes it less likely that CF cell-specific changes are a key factor in the development of Pa infection [15] [22].

Dysregulation of the host inflammatory response has been postulated as the putative basic defect in cystic fibrosis. Support for this hypothesis lies in the fact that abnormally high concentrations of inflammatory mediators are seen in cystic fibrosis cell cultures and uninfected *ex vivo* tissue samples [23] [24] [25] [26]. Furthermore, findings from lung lavage studies show that inflammation is present in children as young as 4 weeks of age who are apparently free of infection [27]. An increase in proinflammatory molecules such as interleukin 8 (IL-8), interleukin 6, tumor necrosis factor alpha (TNF- α), and arachidonic acid metabolites has been found in patients with cystic fibrosis [28] [29] [30]. Stimulation of the nuclear factor-κB pathway, platelet hyper-reactivity, and abnormalities in neutrophil apoptosis have also been reported [31] [32] [33]. At the same time, concentrations of native anti-inflammatory substances such as interleukin 10, lipoxin, and docosahexaenoic acid are reduced [25] [28] [34] leading to an imbalance between proinflammatory and anti-inflammatory mediators that favours unabated inflammation. Other evidences suggest that CF airway inflammatory response to

infectious agents is exaggerated and/or prolonged [35]. CF patients have been shown to exhibit larger amounts of neutrophils and IL-8 in bronchoalveolar lavage fluid than non-CF subjects in response to similar levels of infection [36]. Furthermore, CF airway epithelial cell lines produced larger quantities of IL-8 than CFTR-corrected cells in response to IL-1β and TNF- α and to bacterial stimulation [37 38 39]. Whether inflammation is directly related to the CFTR defect is still disputed, but an exaggerated, sustained, and prolonged inflammatory response to bacterial and viral pathogens is an accepted feature of CF lung disease.

Though the debate continues on the pathophysiologic relevance of some of these factors, the bulk of the evidence suggests that dehydration of airway surface liquid is a key factor that impairs cilia functioning and mucociliary clearance in CF, so inhaled bacteria are not cleared [15 40].

Pseudomonas aeruginosa: phenotipic and adaptive genetic changes

Pseudomonas aeruginosa is an opportunistic pathogen that causes pneumonia in individuals whose natural lung defences are compromised, and it is known to significantly contribute to the pathogenesis of cystic fibrosis (CF). It is generally accepted that *P. aeruginosa* is the most clinically important pathogen in CF lung disease. Its prevalence in CF respiratory tract cultures goes from10 to 30% at ages 0–5 years to 80% at ages ≥ 18 years [41]. The presence of *P. aeruginosa* in the respiratory tract and the inflammatory response it elicits are associated with an increase in the rate of deterioration in lung function, and these factors are the leading causes of most of the morbidity and ultimate mortality in CF [40]. Chronic *P. aeruginosa* infection increases the risk of death 2.6 times [42].

Perhaps the most important feature of *P. aeruginosa* is its ability to persist in the CF lung as a result of its tremendous genetic flexibility. Environmental isolates that colonize the airways are motile and express numerous exoproducts (e.g. protease, phospholipases and elastase). The organisms rapidly switch to a more indolent mode of growth, turning off the expression of immunostimulatory

products such as flagella and initiating its biofilm mode of growth; this adaptation plays a critical role during chronic infection with *P. aeruginosa*.

Early *P. aeruginosa* isolates are usually non-mucoid, motile and highly susceptible to antibiotics, suggesting they are usually acquired from the environment [41] [43], although recent outbreaks of so-called 'epidemic' strains being passed from patient to patient suggest some isolates might acquire an enhanced transmissibility, although the basis for this is unknown [44]. The most profound increases in the rate of lung function decline occur when *P. aeruginosa* undergoes phenotypic and genotypic changes. *P. aeruginosa* isolates from the lungs of patients with CF are quite distinctive from those causing acute infection in other settings. These characteristics are not present in isolates causing initial colonization but appear to be selected within CF airways and occur increasingly with length of lung infection. Whereas early isolates appear much like environmental isolates in their phenotype, later isolates are more resistant to antibiotics and frequently mucoid [45]. Mucoidy is a descriptive term for the overproduction of the exopolysaccharide alginate, which is a negatively charged, linear copolymer of partially O-acetylated b-1,4-linked D-mannuronic acid and its C5 epimer, a-L-guluronic acid [46]. Additional phenotypic changes seen in CF isolates of *P. aeruginosa* include the loss of O-side chains on LPS making the strains non reactive with typing sera [47], distinctive acylation of LPS [48], loss of flagella-dependent motility [49], and increased auxotrophy [50]. Moreover these bacteria adopt a biofilm mode of growth *in vivo*. Biofilms are sessile communities of bacteria that form in aggregates on surfaces using a hydrated polymeric matrix of their own synthesis. Some common clinical characteristics of biofilm infections have been identified: slow growth of organisms, stimulation of production of antibodies that are ineffective in clearing bacteria, inherent resistance to antibiotics, and an inability to eradicate biofilm infections even in hosts with intact immune systems [51].

Together, alginate production and biofilm formation by mucoid strains of *P. aeruginosa* contribute significantly to the resistance of these organisms to treatment regimens and host defences, resulting in a poor prognosis for the CF patient [52]. The steps of biofilm formation include initial attachment

to a surface such as mucin-covered epithelial cells by free-swimming or planktonic bacteria, microcolony formation, development of a mature biofilm and release of planktonic organisms to begin the cycle anew [53] [54] (Fig. 3). Secretion of an exopolymeric substance (EPS) occurs after initial attachment and continues throughout biofilm formation. However, questions remain as to precisely when alginate is expressed in relation to the production of other polysaccharides or matrix material in the course of CF lung infection. In addition to exopolysaccharides, the biofilm matrix contains a significant amount of nucleic acid [55] [56]. There remain significant gaps in our understanding of how *P. aeruginosa* survives the inflammatory environment of the lung before stable mucoid conversion (Fig. 3, steps 1–3). One must consider that during initial colonization, biofilm formation in the CF lung probably precludes the switch to mucoidy. An intriguing idea is that these polysaccharides and nucleic acids contribute to the matrix EPS of biofilms formed by early colonizing non-mucoid *P. aeruginosa* strains, before the conversion to alginate-producing variants (Fig. 3, step 3). Although alginate does not appear to be required for biofilm formation by non-mucoid strains *in vitro*, alginate production (especially in its O-acetylated form) appears to contribute significantly to the biofilm architecture. An alginate overproducing *P. aeruginosa* strain forms highly structured biofilms on an abiotic surface as compared with its isogenic, non-mucoid strain. After a delay in biofilm initiation, the mucoid strain produced biofilms with large microcolonies separated by water channels, whereas the non-mucoid strain rapidly attached and initiated growth but did not exhibit the extensive architecture of the mucoid strain [57] [58] [59].

Infection of the CF lung by microorganisms causes inflammatory cells to be recruited to the site of infection, where they release reactive oxygen species (ROS) and cause extensive tissue damage [52]. Alginate appears to protect *P. aeruginosa* from the consequences of this inflammation as it scavenges free radicals released by activated macrophages *in vitro*. Alginate also appears to provide protection from phagocytic clearance and defensins, most probably because it provides a physical and chemical barrier to the bacterium [52].

Alginate production might allow the bacteria to survive and persist better than their non-mucoid counterparts, which are more virulent but also better recognized by immune defences. These 'persistors' might then grow and divide, establishing a chronic infection that is difficult to eradicate. In this way, the balance is tipped in favour of chronic colonization by mucoid strains of *P. aeruginosa* in the CF lung.

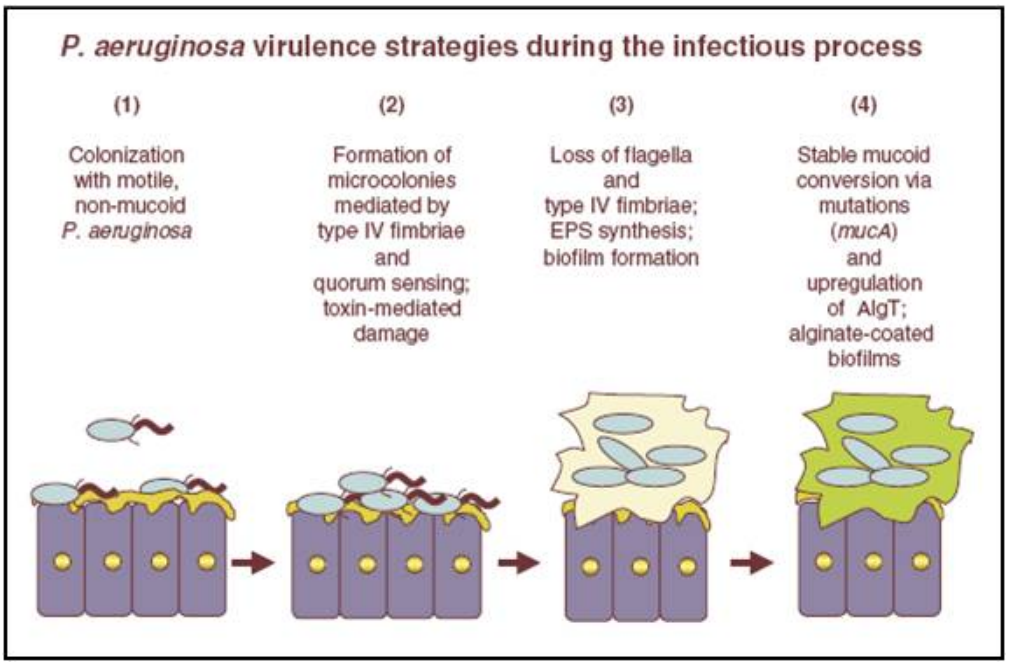


Figure 3. Proposed *P. aeruginosa* virulence strategies during the infectious process. Patients are initially colonized with motile (planktonic), non-mucoid *P. aeruginosa* strains, which attach to a surface such as mucin-covered epithelial cells (step 1). Individual bacteria, which express type IV fimbriae and secrete homoserine lactone molecules in a form of cell-to-cell communication (quorum sensing), aggregate and form microcolonies (step 2). Over time, microcolonies develop into a mature biofilm characterized by secretion of an EPS, loss of flagella and type IV fimbriae, and the formation of three-dimensional structures encasing both aerobically and anaerobically respiring colonies (step 3). Inflammatory cells are recruited to the site of infection, where they release reactive oxygen species (ROS) and cause extensive tissue damage. This applies a selective pressure to the colonizing *P. aeruginosa* strains, leading to *mucA* mutations, deregulation of AlgT and subsequent stable mucoid conversion (step 4). (From Reference 60)

P. aeruginosa has a very large genome—at 6.3 Mbp it is 37% larger than the best-studied bacterial pathogen, *Escherichia coli*, which has a genome size of 4.6 Mbp. With 5570 predicted open reading frames, the genetic complexity of *P. aeruginosa* approaches that of the simple eukaryotic organism, *Saccharomyces cereviseae*. This complete genome offers the potential for a tremendous ability to adapt to multiple different environments, including the CF airway. *P. aeruginosa* isolated from CF

sputa have even larger genomes than the laboratory strain, PAO1, suggesting that they have acquired new genes during their adaptation, in addition to alterations in those already present [60]. Responding to environmental signals during acute infections, *P. aeruginosa*, as other microorganisms, reversibly regulates gene expression, thereby adapting its phenotype accordingly. During the course of chronic infection, however, reversible gene regulation is often lost, leading to the development of mutants that differ genotypically and phenotypically from the originally infecting strain when investigated *in vitro*. Such microevolution has previously been observed in *P. aeruginosa* isolates of a limited number of chronically infected CF patients [61 62 63 64], including loss-of-function mutations, acquisition or loss of genomic islets/islands, genome rearrangements, recombination, or point mutations, in a surprisingly large number of genes. The progressive microevolution of *P. aeruginosa* in patients with CF has been interpreted as an *in vivo* selection process, resulting in less virulent variants, which consequently do less harm to its host than the original colonizing strain [63 64]. Virulence can be defined in terms of establishment-of-infection assays, in which the loss of a virulence factor results in a decrease in the ability to cause acute disease, it also includes the capacity of the pathogen to persist in a given host, causing chronic infection. The expression of virulence factors needed to induce acute infection is reduced or even lost in long-term *P. aeruginosa* isolates from patients with CF suggesting that adaptation promotes selection of less invasive phenotypes as well as favors factors needed for persistent infection.

Pa isolates from environmental and clinical habitats are equipped with a similar repertoire of virulence mechanisms and pathogenicity factors for induction of acute infections, unless microevolution in a particular habitat, such as the CF lungs, selects for new clonal variants. However, new clonal variants do not differ in virulence from strains isolated earlier from CF lungs or from the environment with regard to their capacity to establish and maintain chronic infection [65]. Therefore the microevolution of *P. aeruginosa* strains within CF lungs leads to populations of genotypes/phenotypes with distinct pathogenic potential, which differ from that of early isolates but are not necessarily less virulent for the CF host. Bacterial microevolution generates diversity that

protects communities from unstable environmental conditions, as those present in the lung of patients with CF with its differential supply of nutrients, oxygen, and exposure to host defense and antimicrobials; inversely, heterogeneous micromilieu tend to maintain diversity to allow persistence of bacterial strains in a specific niche.

Pseudomonas aeruginosa: establishment of chronic infection

CFTR is proposed to be a receptor for *P. aeruginosa* binding to airway epithelium for subsequent phagocytosis and clearance by desquamation [66] [67]. The diminished or non-existent binding of *P. aeruginosa* to the CF epithelium leads to a reduced initial clearance, allowing the organisms sufficient time to take advantage of the dehydrated ASL and remain within the airway lumen by binding to mucins via the bacterial FliD protein [68]. It is postulated that this mechanism is important in the initiation of endobronchial infection. Subsequently, the microbial cells survive and grow within a hypoxic environment [15] [69], wherein increased production of alginate occurs [3] [70], further serving to protect the microbe from host defenses.

A schematic model of the pathogenic events hypothesized to lead to chronic Pa infection in airways of CF patients is outlined in Figure 5. This sequence consistent with several aspects of the low-volume hypothesis.

First, CF airway epithelia excessively absorb Na^+ and Cl^- (and water) from the lumen, deplete the periciliary liquid layer (PCL), and slow/abolish mucus clearance (Fig. 5a and 5b) [12] [71] [72]. Accelerated Na^+ absorption, which reflects the absence of CFTR's normal inhibitory activity on ENaC, is fueled by an increased turnover rate of ATP-consuming Na^+-K^+-ATPase pumps leading to two- to threefold increases in CF airway epithelial O2 consumption [73]. Second, despite the failure to clear mucus from airway surfaces, goblet cells likely continue to secrete mucins and generate plaques and plugs on CF airway surfaces (Fig. 5c). It is anticipated that the combined defects of excessive volume absorption and continued mucin secretion increases the concentration of mucins within of the adherent plaques / plugs, generating mucus contents of about 15–20%, as reported *in*

vivo. Thus, the combination of the increased O_2 consumption by the CF epithelium, coupled with the deep mucus plaques forming on airway surfaces that restrict O_2 diffusion (Fig. 5c, blue color in bar), creates steep O_2 gradients and hypoxic niches within the adherent mucus plaques and plugs. Third, bacteria deposited on thickened mucus can penetrate into hypoxic zones (Fig. 5d). When the normal rotational mucus transport ceased due to excessive volume absorption, the vertical "currents" within transported mucus are abolished, but motile *P. aeruginosa* still penetrate thickened mucus [15]. Note that environmental *P. aeruginosa* strains such as those that characterize early infection are motile and would likely penetrate mucus readily and evade host neutrophils and macrophages, which appear unable to penetrate thickened mucus. Fourth, *P. aeruginosa* can grow in hypoxic/anaerobic CF mucus (Fig. 5e). In part, growth under anaerobic conditions may be supported by the terminal electron acceptor, nitrate (~20 μM), contained in ASL. Importantly, they exhibit adaptations consistent with biofilm formation, e.g. alginate production. The increased alginate formation may represent a stress response to hypoxia that is part of the process that forms biofilmlike macrocolonies, the predominant phenotype of *P. aeruginosa* in CF airways. Finally, the capacity of *P. aeruginosa* to proliferate in hypoxic mucus will generate fully hypoxic (anaerobic) conditions in patients with persistent CF airways infection (Fig. 5f, blu bar).

The reduced O_2 tension in the mucopurulent intraluminal contents of CF airways may, therefore, be one variable contributing to the persistence of *P. aeruginosa* macrocolonies in CF airways. The consequences of the macrocolony growth state include resistance to antibiotics [40] and host phagocyte killing (Fig. 5f), all of which contribute to the persistence of *P. aeruginosa* infection and the chronic destructive airways disease characteristics of CF.

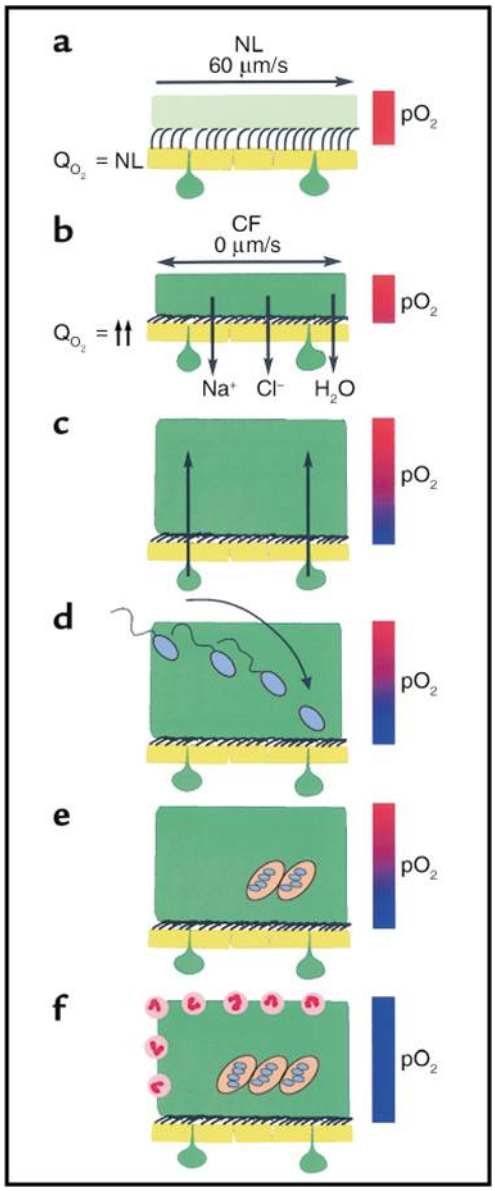


Figure 4. Schematic model of the pathogenic events hypothesized to lead to chronic *P. aeruginosa* infection in airways of CF patients. (**a**) The presence of the low-viscosity PCL facilitates efficient mucociliary clearance (denoted by vector). A normal rate of epithelial O_2 consumption (QO_2; left) produces no O_2 gradients within this thin ASL (denoted by red bar). (**b**) Excessive CF volume depletion (denoted by vertical arrows) removes the PCL, mucus becomes adherent to epithelial surfaces, and mucus transport slows/stops (bidirectional vector). The raised O_2 consumption (left) does not generate gradients in thin films of ASL. (**c**) The raised CF epithelial QO_2 generates steep hypoxic gradients (blue color in bar) in thickened mucus masses. (**d**) *P. aeruginosa* bacteria deposited on mucus surfaces penetrate actively and/or into hypoxic zones within the mucus masses. (**e**) *P. aeruginosa* adapts to hypoxic niches within mucus masses with increased alginate formation and the creation of macrocolonies. (**f**) Macrocolonies resist secondary defenses, including neutrophils, setting the stage for chronic infection. The presence of increased macrocolony density and, to a lesser extent neutrophils, render the now mucopurulent mass hypoxic (blue bar). (From Reference 15)

Pseudomonas aeruginosa: secreted virulence determinants

The wealth of literature describing the mechanisms of virulence and pathogenesis of *P.aeruginosa* reflects their reliance not on a single virulence factor, but rather the precise and delicate interplay between different factors leading from efficient colonization and biofilm formation to tissue necrosis, invasion and dissemination, as well as activation of both local and systemic inflammatory responses. The numerous virulence factors are associated with the ability of Pa to adhere to host cell surfaces, to form biofilms and to secrete hydrolytic enzymes and toxic compounds. Expression of the virulence factors is controlled by several complex cascades that include quorum-sensing (QS)

and two-component-system networks. QS is a mechanism shared by Gram-negative bacteria that allows bacteria-to-bacteria cell signaling through small molecules, acyl homoserine lactones (AHL), also known as autoinducers that diffuse freely across bacterial membranes. When a certain bacterial density or "quorum" is obtained, these molecules reach a threshold concentration at which, as cofactors of transcriptional regulators, they allow coordinated gene expression expression in an entire bacterial population. This coordinated gene expression concerns survival genes and more importantly, genes coding for virulence factors and biofilm formation. There are two such QS systems in *P. aeruginosa*, las and rhl that interact in a hierarchical manner, the first activating the second and both following similar overall pathways. Briefly, a gene encoding an autoinducer synthase ("I" genes, *lasI* or *rhlI*) is activated, the synthesized autoinducer (oxododecanyl or oxohexanoyl-homoserine lactone) diffuses into the environment, reaches a threshold concentration allowing it to bind to transcriptional activators forming a complex that activates, among others, genes coding virulence factors such as elastase, ExoA, type II secretion system apparatus proteins, alkaline protease, alginate, pyocyanin and pyoverdine.

Pseudomonas Proteases

Major virulence factors produced *by P.aeruginosa* include secreted proteases that damage host tissues. One of the best characterized *Pseudomonas* proteases is Elastase B, or lasB, that is a metalloproteinase secreted into the extracellular space through a type II secretion system. Elastase has been shown to have a role in the pathogenesis of *P. aeruginosa* respiratory infections by rupturing the respiratory epithelium through tight-junction destruction, thus increasing epithelial permeability and facilitating neutrophil recruitment [74]. *P. aeruginosa* elastase can also decrease host immune response through cleavage of respiratory tract surfactant proteins A and D and Proteinase-activated receptor 2 into inactive forms [75] [76]. This protease can also inactivate host inflammatory cytokines such as TNF- α and interferon-γ (IFN- γ) [77].

Other proteases secreted by *P. aeruginosa* such as protease IV also have a role in pathogenesis. Although, protease IV is particularly known to participate in the pathogenesis of *P. aeruginosa* keratitis [78], it has only recently been established that protease IV is also involved in the pathogenesis of lung infection through degradation of surfactant proteins A, D and B [79].

Alkaline protease (APR) is a zinc metalloprotease which is similar to the protease secreted by *Serratia marcescens* and has been classified in the serralysin family. This fibrin lysing protease is secreted by Pa through a type I secretion system [80]. Expression of APR depends on the apr genes [81]. In contrast to most proteins secreted from *P. aeruginosa*, which are translocated to the extracellular medium by a two-step mechanism, APR employs an independent secretion pathway with a one-step mechanism without an N-terminal signal peptide [82]. Although its pathogenic role is only clear in corneal infections as is the case for most *P. aeruginosa* proteases, it may participate in the pathogenesis of acute lung injury. This is known to modulate inflammatory and immune responses by altering the bioavaibility of cytokines. APR is reported to degrade IFN- γ [83] and "regulated on activation, normal T cells expressed and secreted" (RANTES) [84], thereby decreasing the bioavailability of these cytokines.

Type III secretion system

Type III secretion systems (TTSS) are shared among *Yersinia*, *Salmonella*, *Shigella* and *Pseudomonas* species as a mechanism to directly inject toxins into the host cells. The type III secretion system of *P. aeruginosa* is a complex pilus-like structure allowing the translocation of effector proteins from the bacteria, across the bacterial membranes and into the eukaryotic cytoplasm through a needle-like appendage forming a pore in the eukaryotic membrane [85]. There are four known toxins, variably expressed in different strains and isolates, injected into host cells by Pa through the TTSS: ExoY, ExoS, ExoT and ExoU.

ExoU is a phopholipase [86] and is correlated with acute cytotoxicity in epithelial cells and macrophages, and contributes to injury in models systems. ExoY is an adenylate cyclase, which

elevates the intracellular cAMP levels in cultured mammalian cells and causes actin cytoskeleton reorganization [87]. ExoS and ExoT are similar, yet distinct, possessing N-terminal RhoGAP and C-terminal ADP-ribosyltransferase domains.

ExoS is a bifunctional cytotoxin with two active domains, a C-terminal ADP-ribosyltranferase domain and an N-terminal Rho GTPase-activating protein (GAP) domain. The ADP-ribosyltransferase activity necessitates a eukaryotic cell cofactor: 14-3-3 protein. The pathogenic role of ExoS is mainly attributable to the ADP-ribosyltranferase activity leading to disruption of normal cytoskeletal organization [88], although GAP activity also plays a similar role [89]. Additionally, it has recently been shown that the C-terminal domain binds to TLR2 and the N-terminal domain binds to TLR4, showing that ExoS may also modulate the host immune and inflammatory response[90].

Macrolides in CF: antipseudomonal effects of azithromycin

The term "macrolide" encompasses a diverse family of unrelated compounds with large macrolactam rings. The macrolide antibiotics consist of 14-, 15-, 16- member macrolactam ring antimicrobials. Erythromycin was discovered in 1952 and is the most widely used macrolide. Azithromycin, clarithromycin and dithromycin are semi-synthetic macrolides similar in structure to erythromycin. Macrolide antibiotics inhibit RNA-dependent protein synthesis by reversibly binding to the 50S ribosomal subunit of a susceptible microorganism. Macrolides are bacteriostatic against *Staphylococcus aureus*, *Haemophilus influenzae* and streptococci, but may be bactericidal in high concentrations. They may also possess anti-pseudomonal activity [91].

By conventional standards *P. aeruginosa* is insensitive to therapeutic concentrations of macrolides; however macrolides have been reported to positively influence the clinical outcome in patients suffering from chronic *P. aeruginosa* infection in diffuse panbronchiolitis [92]. Diffuse panbronchiolitis was first reported in Japan and is characterized by an inflammatory cell infiltration in the respiratory bronchioles, leading to their obstruction and dilatation. As disease progresses,

patients typically become colonized with mucoid strains of *P. aeruginosa* accompanied by cystic changes of the lung and by poor clinical prognosis due to progressive deterioration of respiratory function. The remarkable parallels between diffuse panbronchiolitis and CF led to the question of whether macrolide antibiotics would also be of benefit in patients with CF and to large-scale randomized controlled trials to elucidate the properties of macrolides for chronic *P. aeruginosa* infection of the lung in CF patients [93]. The majority of clinical studies report positive trends concerning the therapeutic potential of macrolide therapy [94]. However, the mechanisms of action in chronic *P. aeruginosa* infection remain obscure [95].

The minimum inhibitory concentration (MIC) of macrolides for *P. aeruginosa* is very high, usually exceeding 500μg/ml. *P. aeruginosa* is equipped with type IV pili, which confer twitching motility once bound to smooth surfaces and to disaccharides of the bronchi. These pili allow single bacterial cells to attach to each other and contribute to the generation of biofilms. Clarithromycin at very low concentrations equal to 0.03× MIC is not able to reduce the production of type IV pili by the bacteria. However, using electronic microscopy it was evident that clarithromycin inhibits the assembly of pili, thus restricting twitching motility and the subsequent formation of biofilm [96]. Moreover piliated *P. aeruginosa* exposed to erythromycin have decreased adherence to acid-injured mouse tracheal epithelia compared with bacteria exposed to other antibiotics [97]. Macrolides may reduce biofilm formation by inhibiting the exopolysaccharide alginate production by guanosine diphospho-D-mannose dehydrogenase [98] or by preventing fimbriae dependent, twitching motility [95]. Flagellin expression, which is the major component of the bacterial flagellar filament and enables *P. aeruginosa* motility is reduced by macrolides [99].

Azithromycin (AZM) is an azalide which differs from erythromycin by the addition of a methyl-substituted nitrogen atom into the lactone ring (Fig. 5).

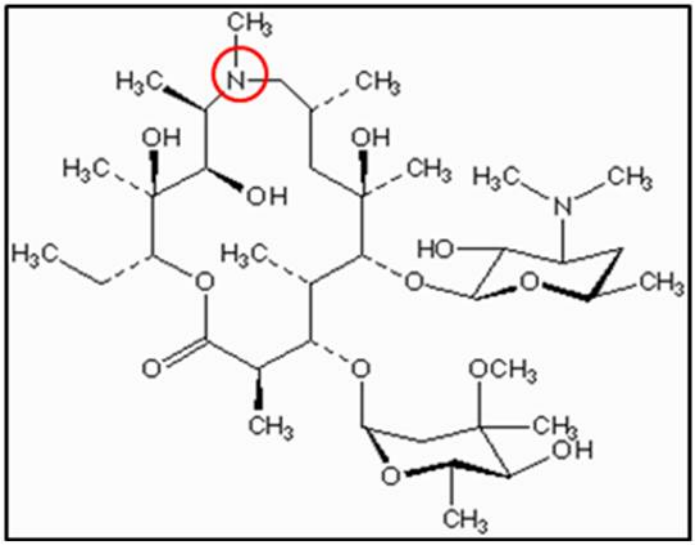


Figure 5. Chemical structure of azithromycin.

These modifications in structure result in better gastrointestinal tolerability and tissue penetration. In addition, there is a decreased risk of interaction with other drugs metabolized by the cytochrome P-450 enzyme system, and increased half-life compared with other macrolides. It has no direct killing effect against the Gram-negative bacteria, *Pseudomonas aeruginosa*, but it is active against other Gram-negative bacteria, such as *Haemophilus influenzae* and *Moraxella catarrhalis*. It has a similar, though less potent, spectrum of activity as erythromycin against Gram-positive bacteria, such as *Streptococci* and *Staphylococcus aureus*.

Following the therapeutic success of macrolide antibiotics in the treatment of patients with diffuse panbronchiolitis chronically infected with *P. aeruginosa*, azithromycin has been studied as a potential therapy for CF patients with chronic *P. aeruginosa* infections [100]. Several randomized clinical trials have evaluated the clinical efficacy of long-term AZM treatment [101] [102]. Most recently, a multi-center trial conducted in the US demonstrated that 6 months of AZM therapy resulted in a significant improvement in lung function, an increase in body weight, as well as a reduction in pulmonary exacerbations and anti-pseudomonas antibiotic use [103].

Although the efficacy of AZM in CF patients with chronic *P. aeruginosa* infection has been well demonstrated, its mechanism of action remains elusive. Several studies have suggested that

macrolides have anti-inflammatory effects that likely contribute to the clinical efficacy of AZM. AZM and other macrolides have been observed to have direct anti-inflammatory effects *in vitro* and in animal studies [104] [105]. AZM may modulate inflammatory pathways by downregulating pro-inflammatory cytokines (such as TNF-α, IL-8, NFkB) and by interfering with neutrophil recruitment and chemotaxis [106] [107] [108]. Although these *in vitro* studies suggest a compelling biological effect, the correlation between clinical efficacy and anti-inflammatory effects in CF patients has not yet been clearly established.

Alternatively, the antibacterial effects of AZM and other macrolides have often been overlooked because AZM is neither bactericidal nor bacteriostatic against *P. aeruginosa* at physiologically achievable concentrations in sputum or serum [109]. However, *in vitro* studies have suggested that at sub-inhibitory concentrations, AZM can decrease the production of bacterial virulence factors such as pyocyanin, elastase, protease and phospholipase C (PLC) [110] [111]. AZM can also interfere with cell-to-cell signaling (quorum-sensing), motility, and biofilm formation, all of which are important in virulence [112] [113] [114] [115]. These effects may directly alter the virulence of *P. aeruginosa* infecting the airways of CF patients, and/or act indirectly by modulating the pro-inflammatory effects of the bacteria.

AIMS OF THE STUDY

Persistence of *P. aeruginosa* in the CF airway involves the emergence of several genotypic and phenotypic changes during bacterial lung colonization.

Pa isolates in CF airways are quite different, not only from isolates in other settings, but also from the prototypical laboratory strain PAO1. Although the PAO1 strain has been highly informative, this laboratory-adapted strain was recovered from a wound infection over 50 years ago and its genome was sequenced a decade ago [116]. It clearly does not represent the Pa diversity of the natural population belonging to the same species and CF colonized patients [117]. Furthermore, while early isolates are quite similar to environmental isolates, the long term colonization of CF airways selects pathoadaptive variants derived from the initially acquired strain [118].

In CF airways *P. aeruginosa* may, at least in part, encounter a low-oxygen environment which develops during infection or as a consequence of biofilm creation [15 52]. Pa strains often grow on the mucus plugs without direct contact with epithelium. However, the product of their metabolism or response to environmental stimuli are released in the extracellular space contributing to the pathogenetic event associated with the presence of Pa. The released proteins have a range of biological functions ranging from host cell toxicity to more subtle alterations of the host cell for the benefit of the invader.

In the attempt to gain information on the identity of the molecules released by Pa strains under aerobic and microaerobic conditions and to identify candidate molecules involved in Pa virulence under comparable *in vivo* conditions, we applied a recently developed shotgun proteomic approach [119] on protein-free conditioned medium obtained from a laboratory (PAO1) and clinical (AA2) strain.

Most studies focusing on CF have investigated specific biomarkers or the transcriptome [120 121 122]. Proteomic analysis has been applied for investigating CF [123 124 125 126 127 122 128], but only a few authors have utilized this approach for investigating proteins found in the extracellular milieu

Proteome analysis of several clinical Pa strains revealed almost identical patterns for the cellular extracts, whereas interclonal diversity and intraclonal diversity were demonstrated for the secretomes of cultured *P. aeruginosa* [129 130].

In the last years, azithromycin a macrolide antibiotic has shown promising results in the treatment of chronic infections by *P. aeruginosa*. In fact, AZM is widely used currently as maintenance therapy in *P. aeruginosa* chronic respiratory infections since several studies have demonstrated its clinical benefit such as improvement in FEV1 and fewer pulmonary exacerbations [102 103].

The improvement in lung function could not be directly correlated with bacterial eradication, suggesting indirect effects of azithromycin on the immunostimulatory capabilities of the *P. aeruginosa* found in the airways of these patients and/or the direct effects on the host immune response. The effect of a macrolide antibiotic on decreasing released exoproducts could diminish the immunostimulatory potential of *P. aeruginosa* in the airways, even without bactericidal activity against the organisms.

In light of the clinical data supporting the use of azithromycin in CF patients, we sought to better characterize the effects of the drug on the proteins released by the Pa clinical isolate AA2 and strains derived from sporadically infected CF individuals and chronically infected CF patients. We postulated that clinically achievable levels of azithromycin could inhibit the expression of *P. aeruginosa* exoproducts, which contribute to infection and the activation of host pro-inflammatory signaling.

Rapid and large-scale identification of Pa released proteins, in particular those associated with the adaptation to the microaerobic environment and proteins associated with strains isolated from long-term colonization in patients, could help to understand which molecules are involved in the intrinsic adaptation ability of Pa and provide a list of possible targets for therapeutic intervention. Moreover this approach could identify potential targets for pharmacological intervention through AZM and explain the clinical benefits associated with macrolide therapy.

MATERIALS AND METHODS

Collection of bacterial conditioned media (CM)

Pa PAO1 laboratory strain and AA2 isolate from a CF patient at the onset of chronic colonization, kindly provided by B. Tummler (Medizinische Hochschule Hannover, Hannover, Germany) [118], were inoculated onto trypticase soy agar (Difco, BD Biosciences) plates and allowed to grow at 37°C overnight. They were then inoculated into modified Vogel-Bonner medium (MVBM) [131] and incubated overnight with continuous agitation.

The day after, Pa cells were diluted in MVBM at a concentration of 1 x 10^8 cfu/ml (OD of 0.1 at 600 nm). The cultures were incubated at 37°C for 16 hours with continuous agitation in aerobiosis or in an anaerobic jar in microaerophilic conditions by adding a sachet containing ascorbic acid as active component (Oxoid, Basingstoke, UK). The Pa cultures incubated in aerobiosis were also exposed to 8 μg/ml AZM (Pfizer, Roma, Italy). The concentration of 8 μg/ml for AZM, which is in the sub-MIC range for P. aeruginosa, is consistent with those described in lungs of patients treated with this macrolide [99 132].

The cultures were then normalized to an optical density of 0.2 OD at 600 nm by adding MVBM. CM from normalized bacterial cultures were collected by centrifugation (7000 g, 30 min, 4°C), and filtered through a 0.22-μm filter to remove any remaining bacteria. The CM were concentrated about 17-fold through centrifugation (800xg, 10 min, 25°C) with Amicon® Ultra-15 30K NMWL centrifugal filter devices (Millipore Corporation, Bedford, MA, USA), precoated with 10mg/ml bovine serum albumin (BSA, Sigma-Aldrich). The samples were then ultracentrifuged at 69400 g for 1 h at 4°C and the CM were subjected to gel filtration by means of PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). CM (now 15X concentrated after the desalting process) were filtered through a 0.22-μm filter and stored at -80°C.

Pa strains, isolated from the airways of CF patients followed up at the Cystic Fibrosis Center of Verona, were cultured as described previously. Written informed consent was obtained from the

patients as it was approved by the local ethical committee. The day after inoculation, these Pa clinical isolates were diluted in TSB at a concentration of 1 x 10^8 cfu/ml (OD of 0.1 at 600 nm) and were incubated at 37°C for 16 hours with continuous agitation in aerobiosis in absence and in presence of AZM at a concentration of 8 μg/ml. Finally the cultures were normalized to an optical density of 0.2 OD at 600 nm by adding TSB, filtered through a 0.22-μm filter and stored at -80°C.

Cell cultures

16HBE14o- AS3 cell line, with CF phenotype (lacking CFTR expression following transfection with an antisense CFTR sequence phenotype, a kind gift from P. Davis (Case Western Reserve University, Cleveland, OH, USA) [133] was grown in Eagle's MEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (Cambrex Bio Science), 1% L-glutamine (Cambrex Bio Science) and 0.4% G418 sulfate (Calbiochem, CN biosciences, La Jolla, CA, USA).

Epithelial respiratory cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. Cells were seeded in a concentration of 4.5 x 10^5 cells/cm^2 and, after 24 hours, were exposed to 10% of CM derived from the AA2 strain for 4 hours prepared as described below.

In the presence of this concentration of CM the cell viability was >95% as determined by the Tripan Blue exclusion test.

Protease digestion of CM

To demonstrate a role for polypeptides in determining the pro-inflammatory response detected in cell lines, the 15X concentrated bacterial CM derived from the AA2 strain grown in aerobiosis was incubated at 37°C overnight with and without concentrations of trypsin (Sigma-Aldrich) ranging from 200 ng/ml to 3.125 ng/ml. The same surnatants were then treated with 250 μM of trypsin inhibitor from Glycine max (Sigma-Aldrich Inc., St Louis, MO, USA) for 1 hour at room

temperature. Finally, the CM was subjected to gelatin/zymography assay to evaluate the extent of protease activity and select the appropriate experimental condition.

RNA isolation, reverse transcription and quantification

Cells were lysed. Total RNA was extracted with the Total RNA Isolation kit (Roche, Germany) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, USA). The reaction was then incubated at 25°C for 10 min and at 37°C for 2 hrs. Relative quantification of gene expression was performed by real time quantitative PCR analysis as previously described [107]. Results were expressed as mean ± standard deviation (SD).

Sample preparation for proteomics analysis

A total of 200 µL of each sample was concentrated to 20 µL and brought to pH 7.9 by the addition of ammonium bicarbonate.

One µg aliquot of sequencing grade modified trypsin (Promega Inc., Madison, WI, USA) was added and the mixture was incubated at 37 °C overnight. The reaction was stopped by acidification with trifluoroacetic acid (Sigma-Aldrich Inc., St Louis, MO, USA) and the sample was desalted and concentrated using PepClean C-18 Spin Columns (PIERCE Biotechnology Inc., Rockford, IL, USA). The eluent was dried in a vacuum system and reconstituted in 5% acetonitrile, 0.1% formic acid.

Proteomics analysis

Trypsin-digested samples were analyzed by two dimensional micro-chromatography coupled to ion trap mass spectrometry (2DC-MS/MS, also referred to as Multidimensional Protein Identification Technology, MudPIT), using ProteomeX-2 system (Thermo Electron Corporation, San José, CA, USA). Briefly, 7 µl of the digested peptide mixture was loaded onto a strong cation exchange column (Biobasic-SCX column, 0.32 i.d. 100 mm, 5 µm, Thermo Electron Corporation, Bellefonte,

PA, USA), eluted stepwise with salt injections of increasing molarity (0, 20, 40, 80, 120, 200, 400, 600, 700 mM) and then captured in turn onto two peptide traps (Zorbax 300 SB-C18, 0.3 i.d. x 5 mm, 5 μm, Agilent Technologies, Palo Alto, CA) for concentration and desalting prior to separation on reversed-phase C_{18} column (Biobasic-18, 0.180 i.d. 100 mm, 5 μm, Thermo Electron Corporation) with an acetonitrile gradient (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile); the gradient profile was 5% eluent B for 5 min, 5 to 65% B in 45 min, 65% B for 3 min and 65 to 95% in 10 min; flow-rate 1 μL/min.The eluting peptides were electrosprayed directly into a LTQ linear ion trap mass spectrometer equipped with a nano-electrospray ionization source (Thermo Finnigan Corp., San Jose, CA, USA). The heated capillary was held at 185 °C; full mass spectra were acquired in positive mode and over a 400–2000 m/z range, followed by five MS/MS events sequentially generated in a data dependent mode on the first to the fifth most intense ions selected from the full MS spectrum scans using dynamic exclusion for MS/MS analysis (collision energy 35%).

Data handling

Using the Bioworks 3.2, based the SEQUEST algorithm (University of Washington, licensed to ThermoElectron Corp.) the experimental mass spectra , were correlated to peptide sequences obtained by matching with the theoretical mass spectra produced from the Pa protein database, downloaded from the NCBI website (www.ncbi.nlm.nih.gov) and updated to October 2008.

The validity of peptide/spectrum matches was assessed using SEQUEST defined parameter thresholds. Spectra or peptide matches were only retained if they had a minimum Xcorr (cross-correlation score) of 1.5 for +1, 2.0 for +2 and 2.5 for +3 spectra and if it had a Δcn (normalized difference in cross-correlation score) of at least 0.08 [134]; a threshold of peptide probability $\leq 1*10^{-3}$ was assigned [135].

Protein lists, obtained from multiple analyses [136], were processed through other software and statistical tools in order to extract further information. In particular, to visualize the protein lists in a

more comprehensible format, we used the MAProMA (Multidimensional Algorithm Protein Map) software [137], which also used for performing qualitative and quantitative comparisons, among different protein lists [138] [139].

Localization and function of identified proteins were defined by using a ftp service (ftp.proteomica.org). Moreover, in order to predict unknown subcellular localizations, we used pSORTb software (http://www.psort.org) [140].

Global cluster analysis was performed using R, a free software environment for statistical evaluation and graphics (www.r-project.org). Full protein profiles, associated to the SEQUEST score, were clustered using the Ward method distance [141] [142].

Protein-protein interactions and gene-gene functional interactions were examined using Cytoscape 2.6.1 (http://www.Cytoscape.org). By means of a cytoscape plugin, Bionetbuilder (http://err.bio.nyu.edu/cytoscape/bionetbuilder/), and STRING 8 (http://string.embl.de/) [143], known interactions were retrieved from several databases such as Prolink, DIP, KEGG, BIND and others.

Gelatin/zymography for metalloprotease activity

The CM utilized were treated as follows: 5 µl of 5x SDS sample buffer (5%SDS, 0.5M Tris-HCl pH 6.8, 25% glycerol) were added to 20 µl of CM. The sample was run on a SDS-PAGE gel containing 1 mg/ml gelatin (Sigma-Aldrich). The gel was washed twice (20 min/cycle) with 2.5% Triton X-100 at room temperature, incubated in 200 ml of activation buffer (10 mM Tris-HCl, 1.25% Triton X-100, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) overnight at 37°C, stained with Coomassie Brilliant Blue G-250 in 20% methanol/ 10% phosphoric acid/ 10% ammonium sulphate and destained in water.

Western blot analysis of exoenzyme S and alkaline metalloprotease precursor

Proteins were precipitated from 12 ml of CM by drop-wise addition of 10% (final concentration) tricloroacetic acid with stirring at 4°C. The sample was then centrifuged at 3000 g for 30 min and

washed 3 times with an excess of an acetone: methanol (8:1) mixture. The pellet was air-dried, resuspended in SDS sample buffer, subjected to SDS-PAGE and Western blotting.

The sample proteins (20 µl per lane) were electrophoresed on SDS-PAGE using 10% acrylamide gel and transferred onto a nitrocellulose membrane (HybondTM ECLTM, Amersham, NJ, USA) [144], using a mini trans-blot apparatus (Bio-Rad, California, USA) following the manufacturer's instructions. To verify equal loading, proteins were separated in 10% polyacrylamide gel containing SDS and stained with Coomassie Brilliant Blue G-250 in 20% methanol/ 10% phosphoric acid/ 10% ammonium sulfate, destained in water and examined for staining intensity. Non-specific binding on the membrane was blocked with 5% dry non-fat milk or 5% bovine serum albumin (BSA, Sigma-Aldrich) in TBS-T buffer (0.2% Tween 20 in Tris-buffered saline pH 7.5) for 1 h at room temperature. The membrane was incubated with a 1:1000 dilution of chicken polyclonal antibody raised against exoenzyme S (Abcam plc., Cambridge, UK) or with a 1:500 dilution of purified rabbit IgG against APR, a kind gift from G. Döring (University of Tübingen, Germany)[145], in TBS-T with 1% BSA overnight at 4°C. The blot was washed four times in TBS-T and then incubated for 1 h at room temperature with goat anti-chicken IgG secondary antibody conjugated to biotin (SouthernBiotech, Birmingham, AL, USA) diluted 1:25000 or donkey anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Amersham, NJ, USA) diluted 1:15000 in TBS-T with 1% BSA. The membrane was washed again four times in TBS-T and finally incubated with streptavidin conjugated to horseradish peroxidase (BioLegend, San Diego, CA, USA) diluted 1:35000 in TBS-T with 1% BSA for 1 h at room temperature. Bound proteins were visualized using the ECL detection system (Millipore Corporation, Bedford, MA, USA).

RESULTS

Effects of PAO1 and AA2 CM on IL-8 mRNA expression in a CF epithelial airway cell line

Looking for potential virulence factors in contact with lung epithelium of CF patients and contributing to the typical inflammatory response, we measured the induction of a pro-inflammatory marker (IL-8) expressed by a CF epithelial airway cell line, 16HBE 14o- AS3, in response to CM derived from Pa strains. Following exposure of the CF cell line to 10% of 15X CM derived from AA2 grown in both aerobic and microaerobic conditions, expression of IL-8 was induced approximately 5.2 and 3.7 times respectively (Fig. 6). The induction of IL-8 mRNA was higher approximately 25% after treatment with CM from AA2 grown in aerobiosis in comparison to the same treatment with CM from AA2 grown in microaerobiosis (Fig. 6). When cells were exposed to CM derived from the laboratory strain PAO1, no statistically significant regulation of expression was detected in both aerobic and anaerobic conditions (Fig. 6). These data indicate that colonization of CF lung by clinical strains is associated with the presence of specific pro-inflammatory molecules in the extracellular milieu.

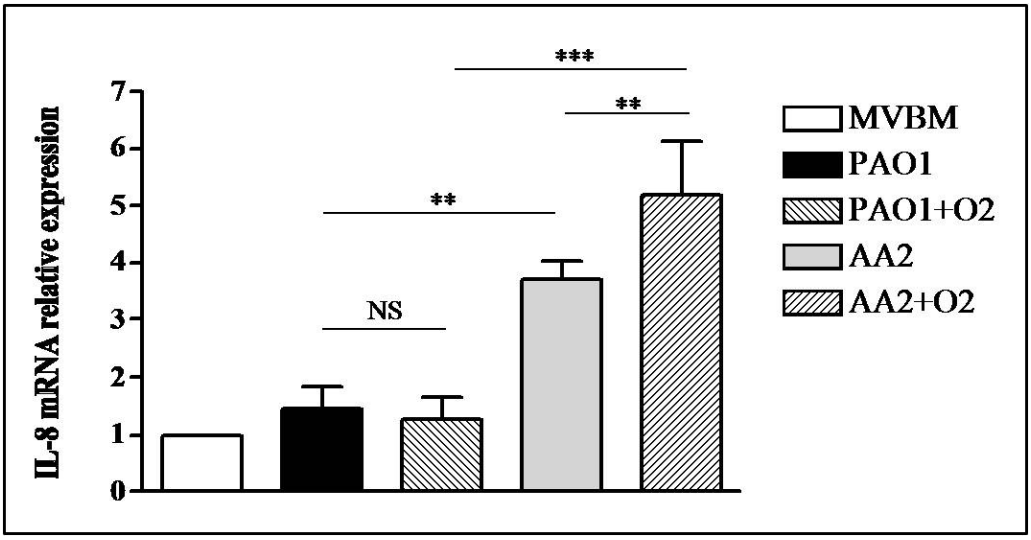


Figure 6. IL-8 mRNA expression. Expression of IL-8 mRNA based on real-time PCR analysis in 16HBE 14o- AS3 cells after treatment with 10% of CM of AA2 and PAO1 grown in aerobic or microaerobic conditions. The values represent the expression levels relative to 16HBE 14o- AS3 treated with 10% of MVBM (means +/- SD). (n=5; *p<0.05, **p<0.01).

Effects of tryptic digested CM on IL-8 mRNA expression in a CF cell line

Many different molecules may be the cause of the pro-inflammatory effect. To evaluate whether polypeptides are involved, we incubated CM overnight at 37°C in the presence or absence of trypsin. Effective protein degradation was evaluated utilizing the decrease of the gelatinase activity, readibly detectable in the AA2 strain, as a marker. Loss of metalloprotease activity in both experimental conditions was detected (data not shown) and was followed by a decreased expression of IL-8 mRNA of about 60% and 50% without and with trypsin respectively (Fig.7). These results indicate that spontaneus autolytic activity occurs, probably due to the presence of MMPs in the CM, and that polypeptides play a relevant role in the induction of pro-inflammatory mediators in the experimental model that we utilized.

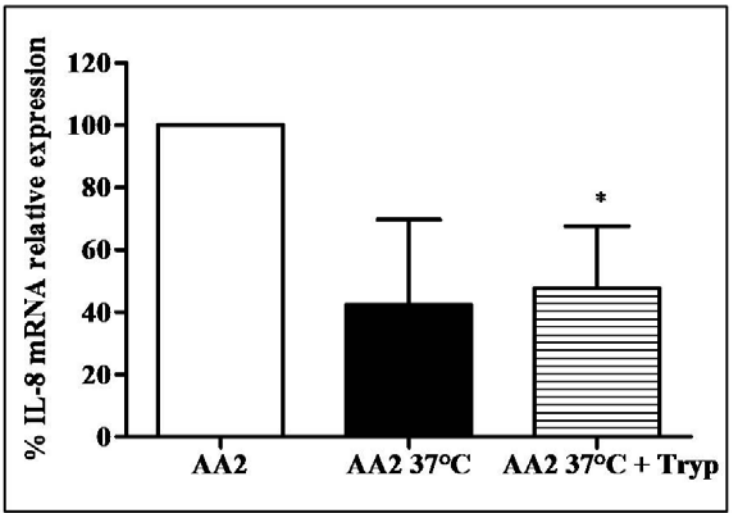


Figure 7. IL-8 mRNA expression after treatment with bacterial CM incubated at 37°C in the absence and presence of the trypsin. Expression of IL-8 mRNA based on real-time PCR analysis in 16HBE 14o- AS3 cell line after treatment with 10% of CM of AA2 grown in aerobic conditions and incubated at 37°C overnight in the absence and presence of the trypsin. The values represent the percentage of expression levels relative to 16HBE 14o- AS3 treated with 10% of AA2 grown in aerobiosis (means +/- SD). (n=3; *$p<0.05$).

Identification of proteins released by PAO1 and AA2 in aerobic and microaerobic conditions

As the former experiments point to the polypeptidic origin of a relevant portion of the pro-inflammatory activity present in the conditioned medium, we studied its composition using a high-throughput proteomic analysis (MudPIT). To identify the maximum number of proteins and to verify the reproducibility of this approach we performed multiple analyses following established procedures [136].

MudPIT analysis identified a total of 451 and 235 proteins in the CM of AA2 and PAO1, respectively, grown in both experimental conditions (aerobiosis and microaerobiosis). More precisely, 75 of 96 (78%) of the proteins identified in PAO1 are shared by the AA2 strain grown in aerobiosis. However, this represents only 33% of the AA2 secretome (75 of 223). In microaerobiosis, these percentages change only slightly to 75 % (104 of 139) for PAO1 and to 45 % (104 of 228) for AA2 (Table 1).

Aerobiosis

In the PAO1 strain cultured in aerobic conditions we identified 96 distinct proteins: 46% of identified proteins were characterized by identification of two or more peptides and 52% of proteins were detected at least 2 times (Table 1). Figure 8A reports the virtual 2D map obtained plotting theoretical pI and MW of identified proteins in CM of PAO1, grown in aerobic conditions. Specifically, the colour/shape code of theoretical spots, permits a rapid evaluation of the identification frequency obtained in seven different individual analyses and a visual description of protein distribution relative to their MW and pI .

Under the same experimental conditions we identified 223 proteins (37% of proteins with two or more peptides and 42% at least two times) in the AA2 strain. The corresponding virtual 2D map is shown in figure 8B.

Comparing the protein lists obtained for the two different strains, 75 proteins result to be shared, whereas 148 and 21 unique proteins were specifically identified in clinical (AA2) and laboratory (PAO1) strains, respectively (Table 1).

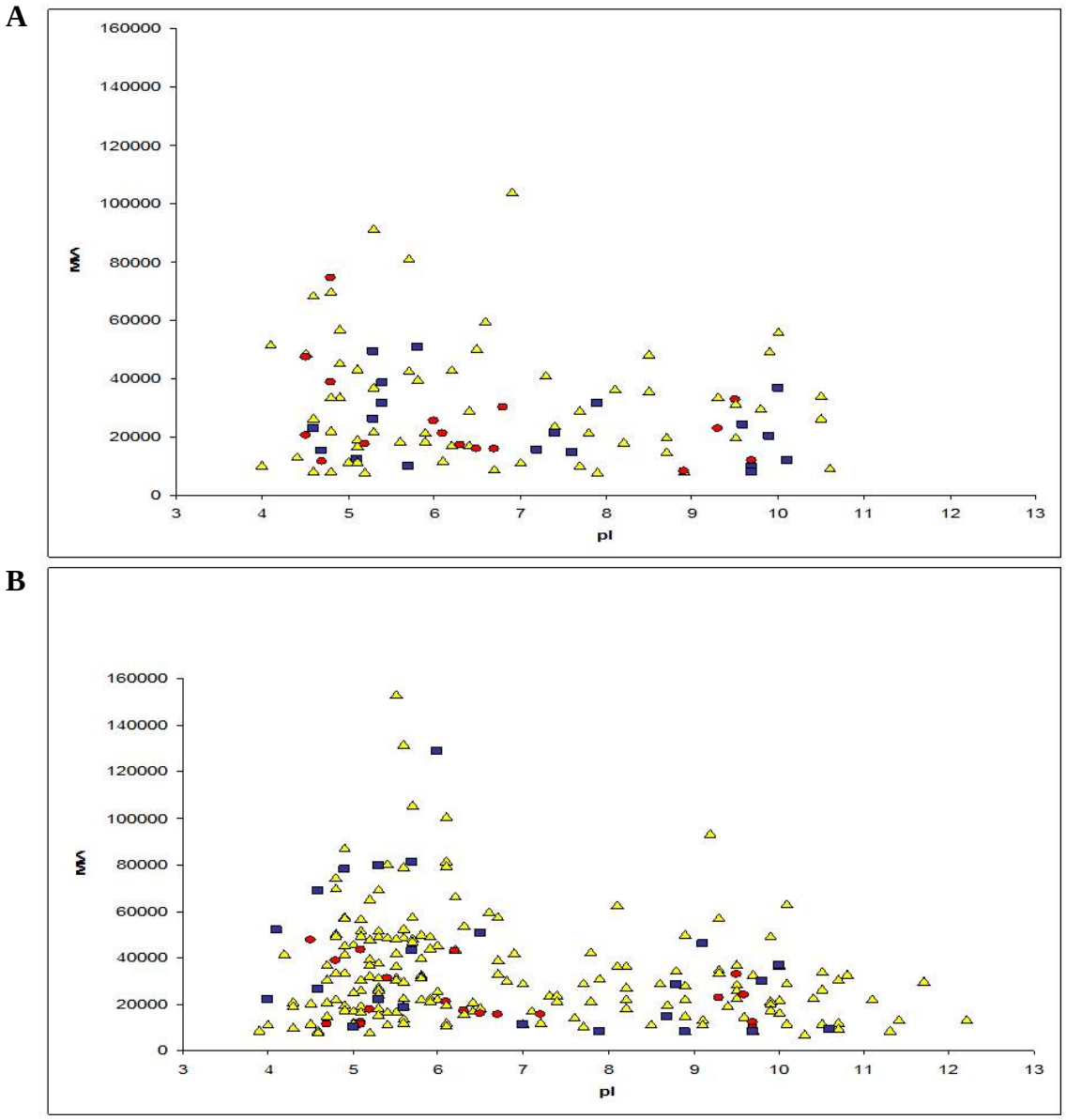


Figure 8. Virtual 2D maps of proteins identified in CM of (A) PAO1 and (B) AA2 grown under aerobic conditions. 2D maps obtained plotting theoretical pI an MW of identified proteins. The color/shape code of theoretical spots is related to the identification frequency from 7 different analysis: yellow/triangles ≤2, blue/squares 3-4 and red/circles ≥5.

Microaerobiosis

During infection or as a consequence of biofilm in CF airways, bacteria may encounter a low-oxygen environment [15 52]. When oxygen is scarce or not available, Pa can utilize alternative external electron acceptors such as nitrate, nitrite or nitrous oxide [15]. For these reasons, with the same approach used in aerobic conditions, we analyzed CM of PAO1 and AA2 strains grown in microaerobic conditions.

Furthermore, 139 and 228 proteins were identified for laboratory (PAO1) and clinical isolated strains (AA2), respectively. In this case, 104 proteins result to be shared, whereas 124 and 35 proteins were distinct for AA2 and PAO1 strains, respectively (Table 1).

For PAO1, 63% of proteins were identified with two or more peptides and 53% were detected at least two times. For the clinical strain, AA2, 40% of proteins were identified with two or more peptides and 45% were detected at least two times.

Among the four different samples, the two strains in both experimental conditions, 61 proteins were found to be shared (Table 1).

	+O_2	**-O_2**	**Shared proteins**	**Total distinct proteins**
PAO1	96	139	78	157
AA2	223	228	147	304
Shared proteins	75	104	61	
Total distinct proteins	244	263		337

Table 1. Total and shared proteins identified by MudPIT proteomic analysis of bacterial CM.

Semiquantitative evaluation of protein expression and hierarchical clustering

The determination of protein differences between two or more biological systems is the most challenging technical task in proteomics. Label-based approaches for protein quantitation are not always practical or feasible. Consequently, more simple alternative approaches were developed to compare protein abundance and are based on label-free quantitation methods [146].

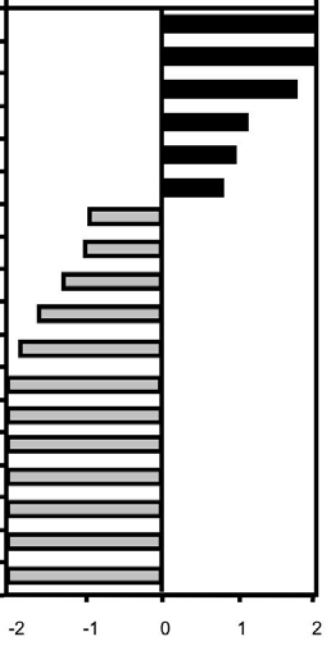
In previous works we have used the label-free approach and demonstrated the correlation between score value, obtained from the SEQUEST algorithm, and relative amount of protein [138] [139]. In this context, specific algorithms, Dave and DCI, have been developed [139]. Other authors have obtained good results using spectral sampling [136], peptide hits [147] or sequence coverage [148].

Based on these findings we compared the lists produced by MudPIT analyses on CM of the laboratory and clinical strains with Dave and DCI (using 0.4 and 400 thresholds, respectively). In particular, under aerobic conditions, 18 proteins resulted differentially expressed between the two strains. Twelve of these were predicted to be up-regulated and 6 down-regulated in the CM of clinical strain (Fig 9A). Applying the same elaboration, under microaerobic conditions, 17 proteins were up-regulated and 5 were down-regulated in the CM of clinical strain (Fig. 9B). Some proteins, such as the flagellar capping protein FliD (GI 15596291) and peptidyl-prolyl cis-trans isomerase A (GI 15598423) were exclusively present in the laboratory strain grown under aerobic conditions (Fig. 9A). Under microaerobic conditions, in addition to flagellar capping protein FliD, other relevant proteins such as flagellin type B (GI 15596289) and branched-chain amino acid transport (GI 15596271) were exclusively identified in the laboratory strain (Fig. 9B).

On the contrary, under aerobic conditions, some proteins resulted to be exclusively present in clinical strain, such as hypothetical protein PA0572 (GI 15595769), hypothetical protein PA1245 (GI 15596442), chitin-binding protein CbpD (GI 15596049), succinyl-CoA synthetase (GI 15596786), hypothetical protein PA3351 (GI 15598547) and hypothetical protein PA3931 (GI 15599126). Finally, under microaerobic conditions, electron transfer flavoprotein (GI 15598147) resulted to be exclusively present in the clinical strain (Fig. 9B).

A

NCBI GI	Protein Name
15596291	Flagellar capping protein FliD
15598423	Peptidyl-prolyl cis-trans isomerase A
15596289	Flagellin type B
15598980	Hypothetical protein PA3785
15595620	Hypothetical protein PA0423
15597400	Probable binding protein component of ABC
15596974	Outer membrane protein OprF precursor
15599933	Hypothetical protein PA4739
15599955	Molecular chaperone DnaK
15599417	Fe(III)-pyochelin receptor precursor
15596446	Alkaline metalloproteinase precursor
15598547	Hypothetical protein PA3351
15596786	Succinyl-CoA synthetase alpha subunit
15599036	Exoenzyme S
15596049	Chitin-binding protein CbpD precursor
15595769	Hypothetical protein PA0572
15596442	Hypothetical protein PA1245
15599126	Hypothetical protein PA3931



B

NCBI GI	Protein Name
15596271	Branched-chain amino acid transport BraC
15596291	Flagellar capping protein FliD
15596289	Flagellin type B
15599882	Ferric iron-binding periplasmic protein HitA
15599902	Hypothetical protein PA4708
15595961	Negative regulator for alginate biosynthesis MucB
15596776	Hypothetical protein PA1579
15596965	Hypothetical protein PA1768
15600682	Thiol:disulfide interchange protein DsbA
15596085	Arginine/ornithine binding protein AotJ
15597810	Outer-membrane lipoprotein carrier protein
15595659	Hypothetical protein PA0462
15597951	Ecotin precursor
15598423	Peptidyl-prolyl cis-trans isomerase A
15595620	Hypothetical protein PA0423
15598277	Hypothetical protein PA3081
15599649	Hypothetical protein PA4453
15598509	Hypothetical protein PA3313
15596974	Outer membrane protein OprF precursor
15599955	Molecular chaperone DnaK
15599036	Exoenzyme S
15598147	Electron transfer flavoprotein alpha-subunit

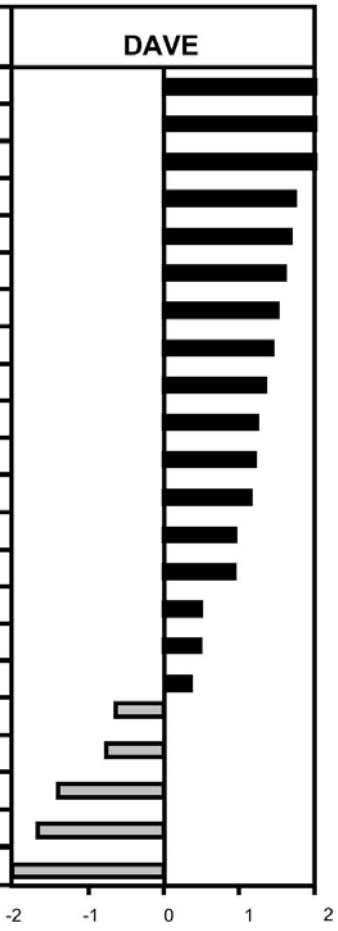
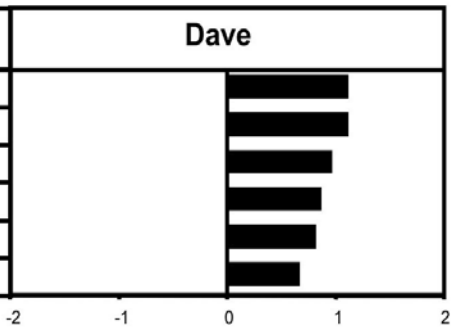


Figure 9. Comparison of differentially expressed proteins in PAO1 versus AA2 in (A) aerobic and (B) microaerobic conditions. Positive Dave values (black bars) correspond to up-regulation in PAO1 versus AA2, negative Dave values (grey bars) indicate down-regulation in PAO1 versus AA2 (7 analyses).

The protein lists of the CM of each strain in the two different conditions were compared. In microaerobiosis 6 proteins were up-regulated in PAO1 (Fig. 10A). In the AA2 strain 10 proteins were modulated (7 up- and 3 down-regulated, fig. 10B). Of note, probable binding protein component of ABC (GI 15597400) and hypothetical protein PA0856 (GI 15596053), presented a similar up-regulation from aerobiosis to microaerobiosis for both laboratory and clinical strains. Several proteases were identified by the MudPIT approach (Table 2).

A

NCBI GI	Protein Name	Dave	DCI
15599882	Ferric iron-binding periplasmic protein HitA	1.1	575
15596053	Hypothetical protein PA0856	1.1	575
15599902	Hypothetical protein PA4708	0.95	675
15597400	Probable binding protein component of ABC	0.85	1400
15598423	Peptidyl-prolyl cis-trans isomerase A	0.8	1200
15600682	Thiol:disulfide interchange protein DsbA	0.65	375



B

NCBI GI	Protein Name	Dave	DCI
15599036	Exoenzyme S	1.6	1025
15596085	Arginine/ornithine binding protein AotJ	1.25	625
15598509	Hypothetical protein PA3313	1.24	1000
15596053	Hypothetical protein PA0856	1.2	750
15598147	Electron transfer flavoprotein alpha-subunit	1.2	800
15595620	Hypothetical protein PA0423	1.	1187
15597400	Probable binding protein component of ABC	0.87	1500
15598547	hypothetical protein PA3351 - FlgM	-1.34	-525
15596446	Alkaline metalloproteinase precursor	-1.5	-3225
15599417	Fe(III)-pyochelin receptor precursor	-1.6	-600

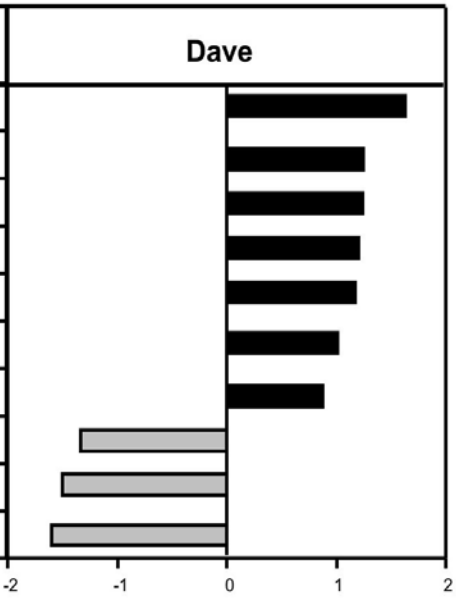


Figure 10. Comparison of differentially expressed proteins in microaerobic versus aerobic conditions for PAO1 and AA2 strains. Positive Dave values (black bars) correspond to up-regulation in PAO1 (A) and/or AA2 (B) grown under microaerobic conditions. Negative Dave values (grey bars) indicate up-regulation in AA2 (B) grown under aerobic conditions (data represent the result of 7 individual analyses).

Table 2. Identification of metalloproteinases and proteases in *P.Aeruginosa CM*. +, identified protein; -, never identified protein.

NCBI GI	Uniprot ID	Gene names	Reference	PAO1	AA2	PAO+O2	AA2+O2
15596446	Q03023	aprA (PA1249)	gi|15596446|ref|NP_249940.1| alkaline metalloproteinase precursor	-	+	+	++++
15597068	P14789	lasA (PA1871)	gi|15597068|ref|NP_250562.1| LasA protease	-	+++	-	+++
15599370	Q9HWK6	prpL (PA4175)	gi|15599370|ref|NP_252864.1| probable endoproteinase (Protease IV)	+	+	+	+++
15598919	P14756	lasB (PA3724)	gi|15598919|ref|NP_252413.1| elastase LasB	-	-	-	++
15595864	Q9I5Q4	PA0667	gi|15595864|ref|NP_249358.1| hypothetical protein PA0667	+	+++	+	++
15600667	Q9HT96	PA5474	gi|15600667|ref|NP_254161.1| probable metalloprotease	-	-	-	++
15596444	Q03025	aprE (PA1247)	gi|15596444|ref|NP_249938.1| alkaline protease secretion protein AprE	-	-	-	++
15595552	Q9I6D8	pfpI (PA0355)	gi|15595552|ref|NP_249046.1| protease PfpI	-	++	+	++
15597951	Q9I088	eco (PA2755)	gi|15597951|ref|NP_251445.1| ecotin	++++	++	+	-
15600417	Q9HTW6	pepP (PA5224)	gi|15600417|ref|NP_253911.1| aminopeptidase P	-	-	-	+
15598453	Q9HYY3	prc (PA3257)	gi|15598453|ref|NP_251947.1| periplasmic tail-specific protease	-	-	-	+
15598522	Q9HYR9	clpP2(PA3326)	gi|15598522|ref|NP_252016.1| ATP-dependent Clp protease	-	-	-	+

Regulation of metalloproteinases and proteases was defined by protein identification frequency on seven specimens for each strain. Alkaline metalloproteinase APR (GI:15596446) resulted overexpressed, in AA2 strain grown under aerobic conditions, by Dave and DCI evaluation.

Although these proteins, except APR, did not pass the Dave and DCI thresholds (passing expressed as ++++), nevertheless, they were identified in the AA2 strain, with a higher identification frequency than in the PAO1 strain (+ to +++). It is of relevance to note that ecotin (GI 15597951), a protease inhibitor, was readily identified in the CM of PAO1 and its peptides were detected with higher frequency under microaerobiosis.

The protein lists were used for unsupervised hierarchical clustering of Pa CM based on their proteomic profiles (Fig. 11). The resulting analysis indicates that the clustering is affected more by strain type than by culture conditions (+/- O_2). In fact, heat map analysis shows that some protein expression traits changed significantly between the two strains rather than in the same strain under different conditions. This result is confirmed by the number of differentially expressed proteins (Figs. 9 and 10).

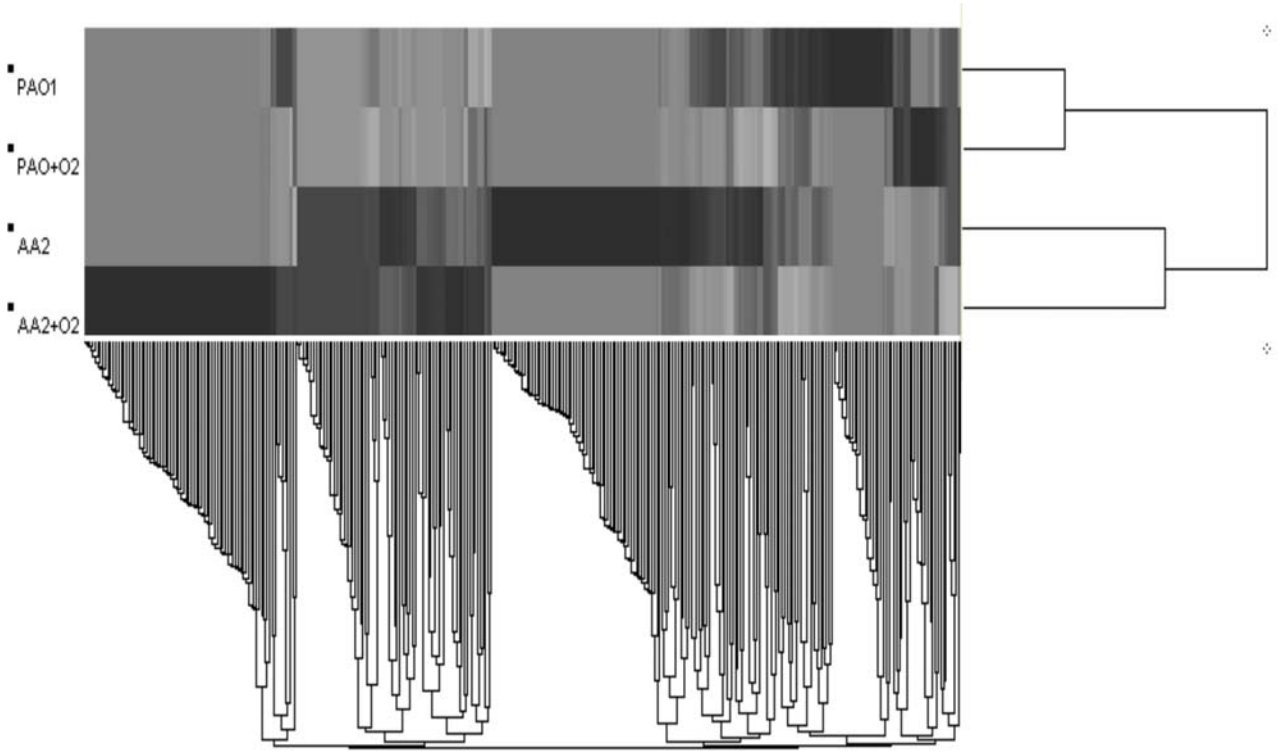


Figure 11. Unsupervised hierarchical clustering of four *Pseudomonas aeruginosa* CM based on their proteomic profile. Hierarchical clustering analysis was based on the SEQUEST score of proteins identified with high confidence in multiple replicate analyses in each experimental condition.

Validation of semi-quantitative data obtained by MudPIT analysis

To confirm the semi-quantitative data obtained from MudPIT analysis we selected two pro-inflammatory candidates (exoenzyme S and the alkaline metalloprotease APR) that feature a different regulation under aerobic versus microaerobic conditions.

From MudPIT analysis the results show that, in aerobiosis, exoenzyme S was undetectable in PAO1 and found in AA2 while, in microaerobiosis, exoenzyme S was found in the PAO1 strain and upregulated in AA2 (Fig. 9). Western blot analysis fully confirms these findings (Fig. 12, lane A).

Members of the metalloprotease family of enzymes were poorly expressed by the PAO1 strain. The AA2 strain appears to express a much larger set of proteases and increase their expression under aerobic conditions (Table 2). Functional assay (zymography) and Western blot analysis for APR, one of the metalloproteases detected in the CM, fully confirmed the findings made by MudPIT analysis (Fig. 12, lanes B and C).

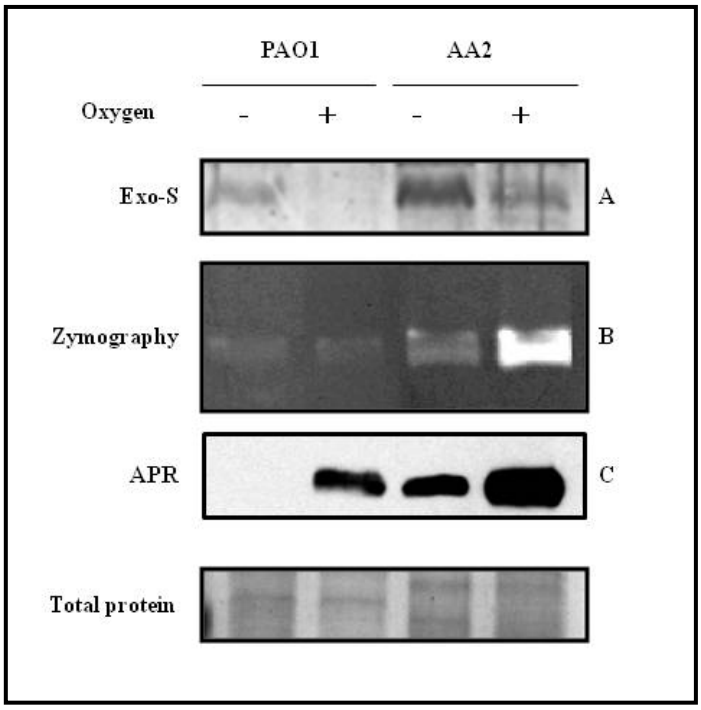


Figure 12. Target validation. Western blotting analysis showing exoenzyme S upregulation in conditoned medium of AA2 grown in microaerobiosis in comparison to aerobiosis (A). Down-regulation of metalloprotease activity and expression in AA2 strain grown in microaerobiosis versus aerobiosis as shown by a zymography assay (B) and Western blot specific for a major Pa alkaline metalloproteinase, APR (C).

Metalloprotease activity in Pa strains correlates with clinical parameters

To evaluate at what extent MMP expression could be associated to specific clinical features we have evaluated a collection of Pa strains, isolated from CF patients featuring sporadic or chronic colonization, for MMP activity. This colonization represents a clinically relevant condition as chronic respiratory infection is a hallmark of CF important for maintaining lung inflammation leading to compromised respiratory function. Since the early description of CF, pulmonary infection has been recognized as having the greatest role in morbidity and mortality leading to premature death in 90% of patients [145]. More precisely chronic colonization was defined as the isolation of at least 3 isolates within 6 months (minimum 30 days interval) while sporadic colonization refer to the isolation of Pa in the bronchial tree in presence or absence of direct or indirect signs of inflammation [145]. Within this context we have evaluated MMP activity in CM derived from 35 isolates defined as chronic and 7 classified as sporadic. All of the sporadic strains release detectable amount of MMP activity while only 11 of 35 (31%) scored positive for the assays among the chronic strains ($p=0.0012$, Fisher's exact test). We then evaluated whether MMP activity was associated to APR expression, the main MMP detected in our analysis. This association was not detected in all the strains positive for MMP activity indicating the presence of other MMPs as the major source of proteolytic activity in some strains (Fig. 13).

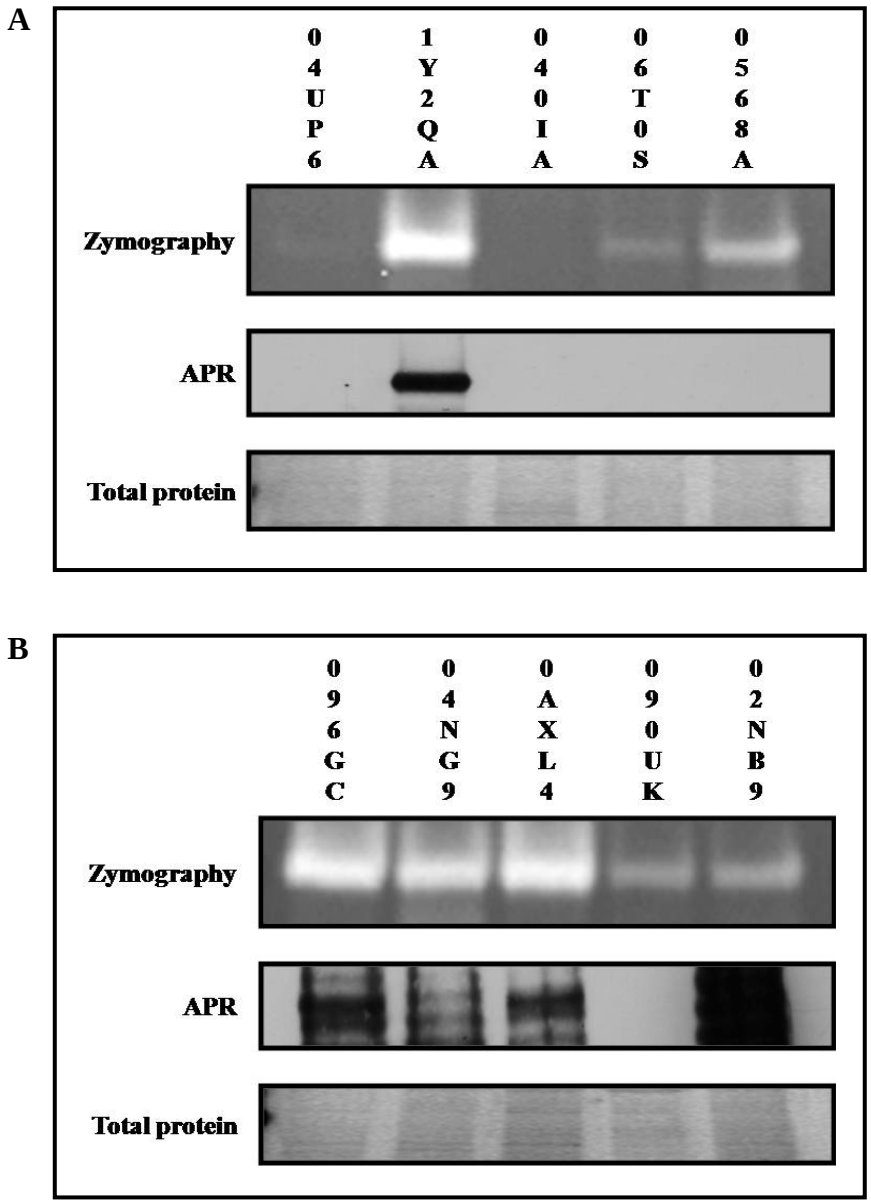


Figure 13. Metalloproteinase activity assay (zymogram) and Western blot in Pa clinical isolates. **Figure 13.** Pa strains were isolated by patients identified as chronically infected (A) or sporadically infected (B) (see methods for description of clinical criteria). Note that anti APR antisera do not react with all the samples showing MMP activity thus suggesting that this activity depends also from MMPs other that APR, the major form identified by MudPIT analysis. More details of the series analyzed is provided in the results section.

Effects of CM from PAO1 and AA2 cultured in presence of AZM on IL-8 mRNA expression in a CF epithelial airway cell line

Many *P. aeruginosa* gene products evoke the epithelial secretion of IL-8, the PMN chemokine that is thought to be especially important in the pathogenesis of CF lung disease. By decreasing the

expression of exoproducts, AZM should also decrease the epithelial expression of IL-8, which is induced by many *P aeruginosa* gene products.

We measured the induction of pro-inflammatory marker IL-8 mRNA expressed by a CF epithelial airway cell line, 16HBE 14o- AS3 in response to CM derived from Pa strains grown in presence or absence of AZM. After exposure of the CF cell line to 10% of 15X CM derived from AA2 and PAO1 grown without AZM we found inductions of expression of IL-8 mRNA of respectively about 4 and 1.4 times (Fig. 14). The induction of these cytokine was about 20% lower after treatment with CM from both AA2 and PAO1 grown in presence of the macrolide compared to those derived from Pa strains grown in absence of AZM (Fig. 14). Based on these data, AZM appears to decrease the bacterial ability to induce the release of pro-inflammatory mediators by CF airway epithelial cells.

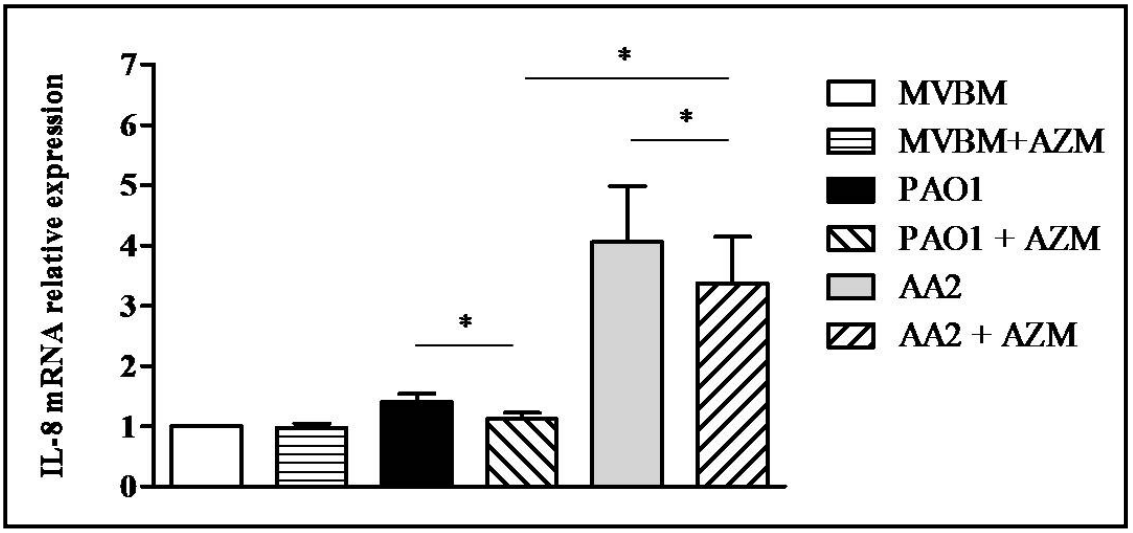


Figure 14. IL-8 mRNA expression. Expression of IL-8 mRNA based on real-time PCR analysis in 16HBE 14o- AS3 cells after treatment with 10% of CM of AA2 and PAO1 grown in absence or presence of AZM (8µg/ml) . The values represent the expression levels relative to 16HBE 14o- AS3 treated with 10% of MVBM (means +/- SD). (n=5; *p<0.05).

Identification of proteins released by AA2 strain grown in absence or presence of AZM

The relative effect of azithromycin on exoproducts expression by the AA2 strain was studied by comparing the proteins present in aliquots of CM from the isolate cultured in absence or presence of the macrolide. In order to attempt to identify candidate factors released by AA2 strain CM, obtained

from Pa after incubation at 37 °C for 16 hours, was digested with trypsin and resulting peptide mixture was analyzed by MudPIT approach. To identify highest number of proteins and to investigate the internal reproducibility of our approach we performed multiple analysis. Comparing the lists produced analyzing by MudPIT technique, for clinical strain AA2 grown without and with AZM, 12 proteins resulted differentially expressed (5 and 7 proteins were up-regulated and down-regulated in CM of clinical strain incubated with AZM, respectively) (Fig. 15).

NCBI GI	Protein Name	Dave	DCI
15598509	Hypothetical protein PA3313	2	2450
15597400	Probable binding protein component of ABC	1.20	3000
15595620	Hypothetical protein PA0423	0.86	1050
15595512	Hypothetical protein PA0315	0.50	800
15599126	Hypothetical protein PA3931	0.33	4800
15596974	Outer membrane protein OprF precursor	-0.67	-600
15596442	Hypothetical protein PA1245	-1	-1600
15598888	Probable outer membrane protein	-1	-400
15596446	Alkaline metalloproteinase precursor	-1.38	-5850
15600115	Azurin precursor	-2	-450
15598725	Probable peroxidase	-2	-800
15595337	Alkyl hydroperoxide reductase subunit C	-2	-450

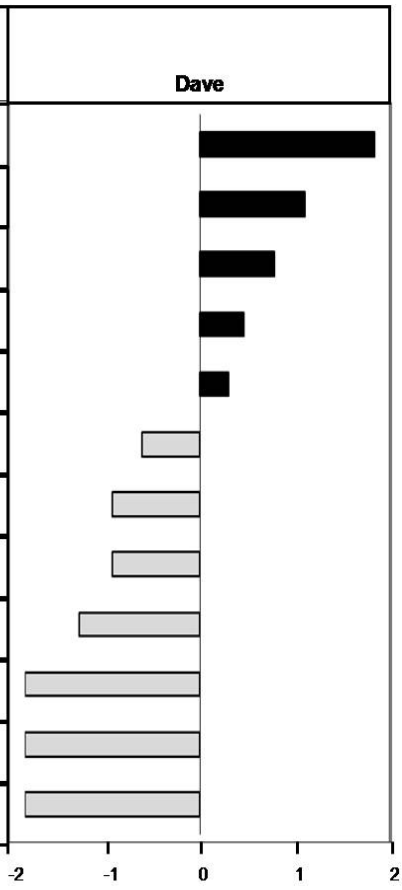


Figure 15. Comparison of differentially expressed proteins in absence of AZM versus presence of the macrolide for AA2 strain. Positive Dave values (black bars) correspond to down-regulation in AA2 grown in absence of AZM. Negative Dave values (grey bars) indicate down-regulation in AA2 grown in presence of AZM (data represent the result of 7 individual analyses).

In particular, we noticed that when the clinical strain AA2 was cultured in the presence of AZM the peptides derived from the alkaline metalloprotease APR were much less represented. Functional assay (zymography) and Western blot analysis for this protease fully confirmed the findings made by MudPIT analysis (Fig 16 A and B).

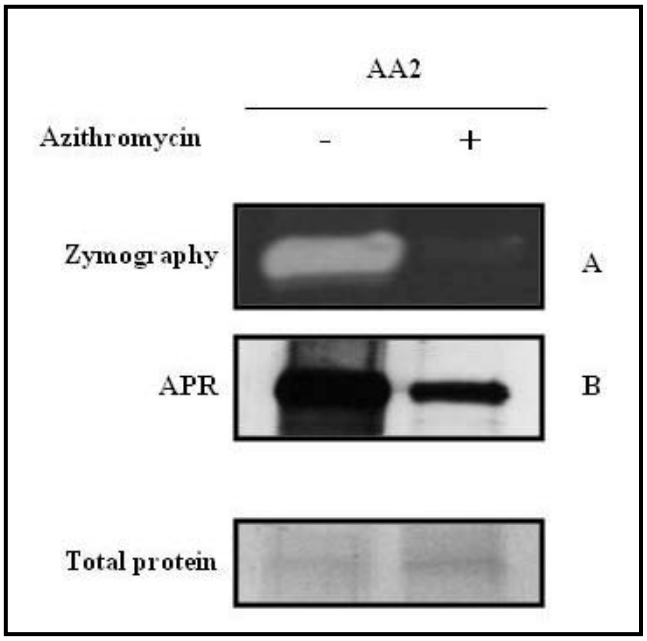


Figure 16. Target validation. Down-regulation of metalloprotease activity and expression in AA2 strain grown in presence of AZM versus absence of it as shown by a zymography assay (A) and Western blot specific for a major Pa alkaline metalloproteinase, APR (B).

Effect of AZM on the metalloprotease activity in Pa clinical strain associated to sporadic and chronic infection in CF patients

We then evaluated the effect of macrolide AZM in a limited series of Pa strains, isolated from CF patients, featuring sporadic or chronic colonization (details of the series analyzed is provided in the results section above). We measured MMP activity (zymography) and alkaline metalloprotease release (western blotting). Within this context we have observed a decreased MMP activity and a strongly reduced release of APR in CM derived from 3 isolates, defined as sporadic, cultured in presence of AZM (Fig. 17B). We then evaluated whether MMP activity and APR release were affected by AZM treatment in CM from other 3 isolates classified as chronic (Fig. 17A). AZM had no effect on these chronic strains. This implies that azithromycin might have the greatest efficacy against early isolates of *P. aeruginosa* in CF, than against the strains from patients with longstanding infection.

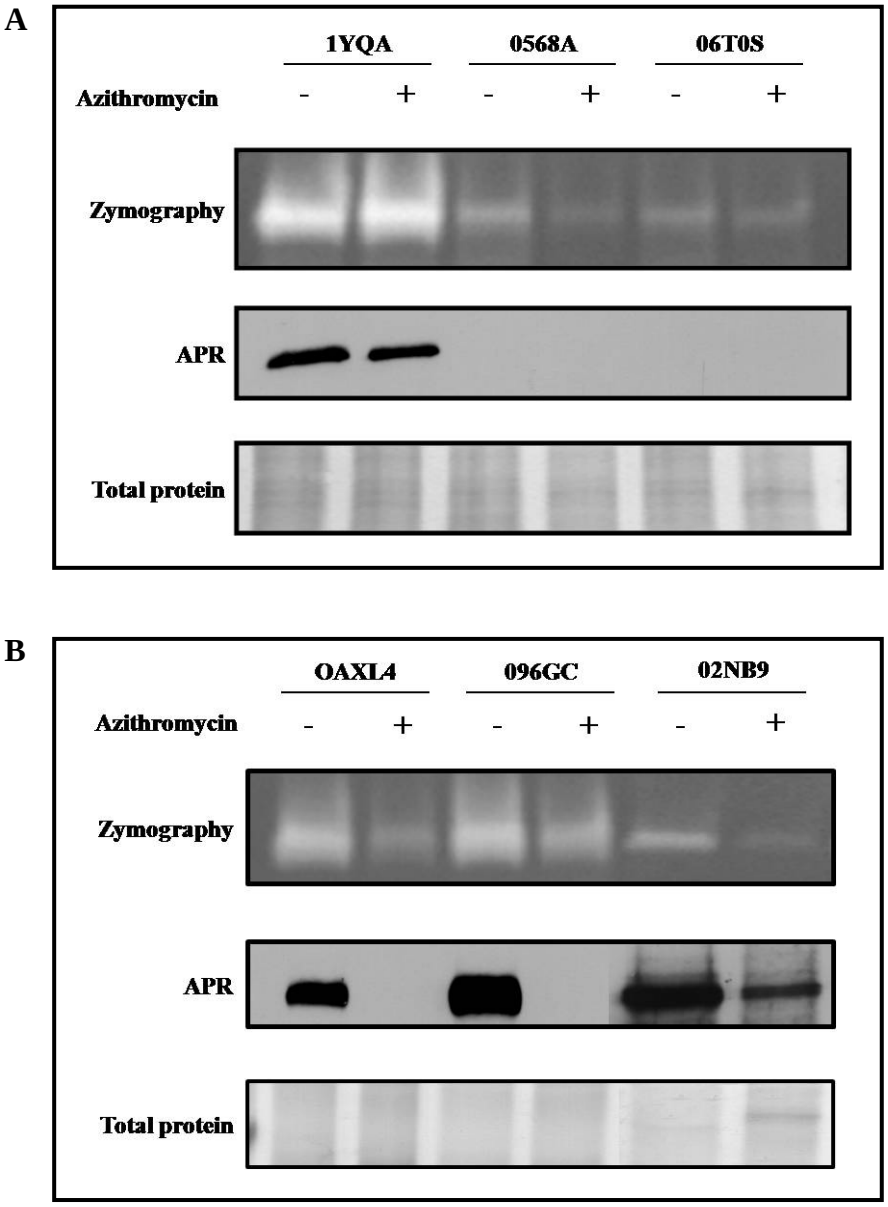


Figure 17. Metalloproteinase activity assay (zymogram) and Western blot in Pa clinical isolates grown in absence or in presence of AZM: Pa strains were isolated by patients identified as chronically infected (A) or sporadically infected (B). Note that AZM had any effect neither on the gelatinase activity nor the APR release in Pa clinical isolates from CF patients chronically infected (A) while the antibiotic strongly decreased the metalloproteinase activity and APR presence in CM from Pa clinical strain of CF subjects sporadically infected (B).

DISCUSSION

Inflammation in CF is a hallmark of the disease and the causative event associated to the negative outcome of these patients that almost invariably suffer and eventually die because of chronic pulmonary disease. Identification of all the players in this complex scenario is mandatory to approach this lethal disease and develop effective therapeutic protocols. We focused our attention to the role of extracellular mediators of inflammation released by *P. aeruginosa* as we observed that Pa-conditioned medium (CM) derived from the AA2 clinical strain had a pro-inflammatory capability that induce the RNA messenger expression of the pro-inflammatory cytokine IL-8 by CF bronchial epithelial cells. The effect was not due to small molecular weight products as measured by utilizing a CM medium subjected to a 30 kDa cut-off ultrafiltration and a further 10kDa cut-off gel filtration step for buffer exchange. The involvement of polypeptides was demonstrated by the substantial loss of effect when the CM was incubated for autolysis at 37°C or subjected to protease digestion. These results suggest the presence of polypeptidic virulence factors in CM derived from clinical isolates of Pa.

In this work we aimed to describe bacterial released proteins and, possibly, to identify candidate pro-inflammatory mediators among them. In addition, we have evaluated the effect of variable oxygen concentrations on the protein production of a representative clinical Pa strain (AA2), comparing its adaptation to microaerobiosis to that of PAO1, a prototypical laboratory strain. In the studies reported herein, we have also examined the effects of the macrolide azithromycin on *P. aeruginosa* protein synthesis as they may help to explain the documented clinical benefits associated with AZM therapy. For the identification of proteins in the bacterial CM we utilized the MudPIT approach that has been demonstrated to be relatively rapid, sensitive and reproducible [139].

Of interest is the observation that the different number of proteins modulated in PAO1 and AA2 by the change from aerobiosis to microaerobiosis, leads to an almost identical extent of

rearrangement of the proteomic profile in both strains. In fact, 66% (157 of 235) distinct proteins in PAO1 and 67% (304 of 451) in AA2 strains were found modulated by the different growing conditions. Given that the genomic structure and organization of the individual strains can be considered identical on a large scale, it is tempting to speculate that a similar set of transcription factors is activated by the change of oxygen concentration in the individual strains that may consequently act on a larger set of unrepressed promoters present in the AA2 strain.

Altogether these results indicate that Pa clinical strains likely utilize a more complex protein synthesis program in comparison with the laboratory strain and underline the importance of using appropriate cellular models for translational studies, specifically in the field of biomarker discovery.

Based on a literature search, potentially interesting candidates to act as pro-inflammatory molecules that are found expressed in the CM of the clinical strain are exoenzyme S (GI 15599036), alkaline metalloproteinase precursor (GI 15596446), hypothetical protein PA1245 (GI 15596442), and outer membrane protein OprF precursor (GI 15596974) involved in transport (such as the arginine/ornithine binding protein AotJ). Other proteins, such as flagellar capping protein FliD (GI 15596291) and flagellin type B (GI 15596289), appear to be involved in the mechanims of motility and/or attachment and have been previously detected [150]. AprX (synonym of PA1245) gene is contiguous to the genes encoding the three proteins constituting the ABC exporter of the alkaline protease, aprA. This gene belongs to the same transcriptional unit as the aprD, aprE and aprF genes and encodes a new protein of unknown function, which can be secreted by the alkaline protease secretion apparatus when expressed in *Escherichia coli* [81 151].

Other proteins identified are involved in metabolic pathways, in post-translational modifications, in adaptation and in the secretion/export apparatus.

Some of these proteins are known to be localized in the membrane or in the intracellular compartment as well as in the periplasm. To rule out the possibility that cell lysis processes associated to the experimental growing conditions [152] could explain the differential protein release detected in the CM, we utilized CM obtained from cells grown at the same optical density, during

the exponential growing phase. We have also verified that this value correlates with the same number of colony-forming units per millilitre (two different experiments, data not shown). Therefore a different degree of cell lysis/death in the different samples cannot explain the different number of proteins identified by MudPIT analysis.

Semi-quantitative evaluation of protein profiles in the different experimental conditions using in-silico analysis identified several candidates as differentially expressed. In particular, we found exoenzyme S and alkaline metalloproteinase up-regulated in CM of the isolated clinical strain. These data predict an up-regulation for exoenzyme S and down-regulation for alkaline metalloproteinase in microaerobiosis. We focused our initial analysis on these two targets in both strains as they represent interesting candidates for their known capability of inducing the release of pro-inflammatory cytokines.

Exoenzyme S is a virulence factor produced by Pa. This toxin possesses two distinct mechanisms of action. Not only can it be delivered into the cytosol of the target cell by means of a type III contact-dependent mechanism, but it can also interact with the cell by acting as a soluble extracellular factor. In both ways it causes cytotoxicity by inducing apoptosis of T cells, as well as activating monocytes, leading to the production of pro-inflammatory cytokines and chemokines. As a consequence, it contributes to inflammatory responses during Pa infection [153]. A phosphorylation-independent interaction has been reported to occur between 14-3-3 proteins and a C-terminal domain within exoenzyme S [154] [155]. The 14-3-3 proteins are a group of highly conserved intracellular dimeric molecules, expressed in plants, invertebrates and higher eukaryotes. More than 200 14-3-3 binding partners have been found that are involved in cell cycle regulation, apoptosis, stress responses, cell metabolism and malignant transformation [155]. Exoenzyme S was undetectable in aerobiosis but was detected by western blot analysis in the CM of PAO1 grown in microaerobiosis. In the AA2 strain it was found expressed in aerobiosis and up-regulated in microaerobiosis, exactly as identified by MudPIT analysis (Fig.12). Even if the specific expression of exoenzyme S in the AA2 strain makes it an attractive candidate as an inducer of pro-

inflammatory cytokines in our experimental system, its pattern of expression does not correspond to that of IL-8 detected in epithelial cells.

We therefore turned our attention to the metalloprotease family of enzymes, known for their strong proteolytic activities in cells and tissues, where they act on fibrin and elastin. It has been demonstrated that Pa elastase is responsible for the rupture of the tight-junction of epithelium and an increase of permeability, leading to tissue invasion and spreading of bacteria [156]. By means of zymography, we could confirm the predicted increase of proteolytic activity in the CM of AA2 grown in aerobiosis in comparison to microaerobic conditions (Fig. 12 B). Western blot analysis with antibodies specific for APR also confirmed the predicted expression and modulation (Fig. 12 C). Given that a strong induction of MMP activity and APR was detected in the AA2 strain, this result is in agreement with the possibility that this may represent part of the functional response to the aerobic condition by Pa strains.

Functional and physical interactions of the alkaline metalloproteinase family of proteins was reconstructed combining interaction network analysis in combination with expression data (Fig. 18). This approach has recently been emphasized to reveal potential functional interaction between multiple candidate proteins or genes [157] [158] [159]. Within this reconstructed network proteases specifically identified in AA2 CM grown under aerobic conditions are present. In particular, LasA, LasB and Protease IV might play an important role in pathogenesis of *P. aeruginosa* infection through the activation of protease-activated receptor 2 (PAR-2), thereby modulating host inflammatory and immune response [160]. Protein AprE has been reported to be involved in the secretion of alkaline protease [81]. Among the proteins predicted to be part of this interactome map (Fig. 18) we observed hypothetical PA0572 that, like APR protein, was found up-regulated in the AA2 CM under aerobic conditions according to MAPROMA differential analysis. We observed a stronger induction of pro-inflammatory activity by the AA2 CM in comparison to the PAO1 strains grown in both experimental conditions and we also observed a significant increase of the effect when AA2 was grown in aerobiosis, a condition associated to the release of larger amounts of APR

and other MMPs. However, the PAO1 strain still expresses detectable (although lower) amounts of MMPs according to MudPIT and APR-specific western blot analysis. The lack of induction of significant amounts of pro-inflammatory mediators by the PAO1 CM in epithelial cells can be explained by the presence of high levels of ecotin, a dimeric periplasmic protein acting as a potent inhibitor of many trypsin-like serine proteases that has been shown to protect bacteria against neutrophil elastase [161]. This protein was identified in the CM of PAO1 with much higher frequency under microaerobic conditions. We therefore propose that the relative amounts of MMPs and their inhibitors must be taken into account when we evaluate the biological effects of the CM, which reflects more precisely the microenvironment where multiple factors simultaneously act on the cells.

The precise mechanism/s that correlate MMP activity with the expression of pro-inflammatory mediators in epithelial cells remains an open question but the possibility to evaluate in an unbiased manner the complexity of the bacterial proteins released in the extracellular milieu appears critical to appreciate the connection and the final effect of specific compounds.

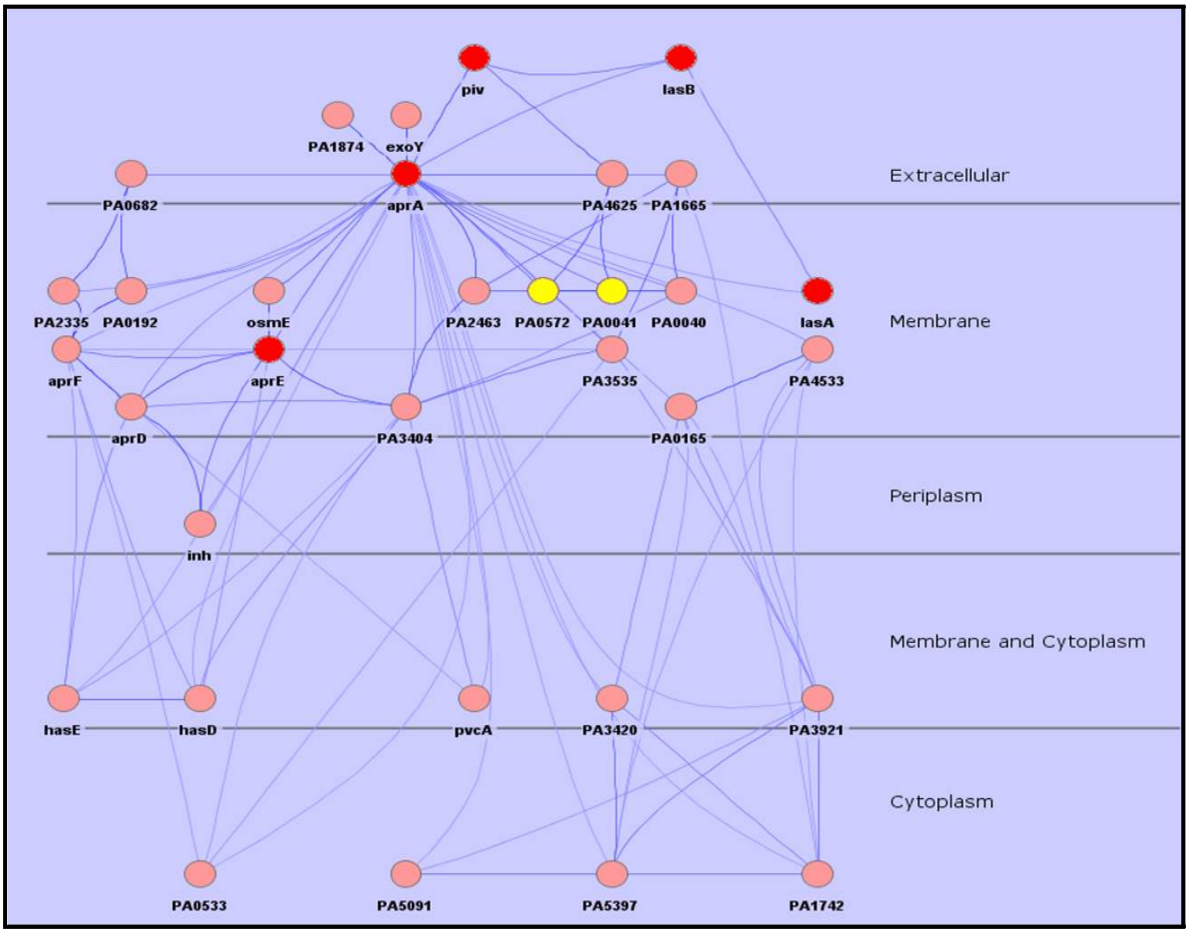


Figure 18. **AA2 metalloproteinase interactome.** In red: proteases identified by the MudPIT approach. In yellow: other proteins identified by the MudPIT approach. In pink: other proteins predicted to be part of the aprA interactome.

In light of the results obtained we studied the effects of the macrolide antibiotic azithromycin on the proteins released from Pa clinical strains. AZM does not act rapidly enough to stop the early stages of bacterial growth, but can interfere with protein synthesis in the late log to early stationary phases. Thus, the organisms are inhibited in their adaptive responses, such as biofilm formation and siderophore expression, as well as in the production of immunostimulatory exoproducts [114].We observed that Pa-conditioned medium derived from the AA2 clinical strain exposed to AZM had a reduced pro-inflammatory capability in comparison with the untreated sample as measured by its capabilty to induce the expression of the cytokine IL-8 by CF bronchial epithelial cells. Inhibition of *P. aeruginosa* exoproduct expression and the subsequent decreased IL-8 production might contribute to the clinical benefits associated with azithromycin therapy in CF patients. As bacteria-

induced airway inflammation is a major cause of pulmonary symptoms in CF patients, the ability of azithromycin to inhibit IL-8 expression and the resultant PMN recruitment could be reflected in improved lung function and fewer pulmonary exacerbations.

Semiquantitative evaluation of protein profiles in AA2 grown in absence and in presence of the macrolide antibiotic identified a dozen of exoproducts as differentially expressed. In particular, we found alkaline metalloproteinase to be down-regulated in CM of the isolated clinical strain AA2 exposed to AZM. Subsequently we observed a decrease of proteolytic activity in the CM of AA2 grown in presence of AZM (Fig. 16 A). Western blot analysis with antibodies specific for APR also confirmed the predicted reduced expression and modulation due to the exposure of AA2 isolate to macrolide (Fig. 16 B). Finally we evaluated the effect of AZM in a collection of Pa strains isolated from CF patients, featuring sporadic or chronic colonization on MMP activity and alkaline metalloprotease release. We have observed a decreased MMP activity and strongly reduced secretion of APR in CM derived from isolates defined as sporadic cultured in presence of AZM (Fig. 17A) while in CM from isolates classified as chronic the MMP activity and APR release were unaffected by AZM treatment (Fig. 17B). Moreover some chronic clinical strains had no metalloprotease activity and detectable level of APR expression. No effects of azithromycin were recorded on clinical strains of *P. aeruginosa* derived from CF patients with established lung disease in comparison to those observed for the strains causing sporadic colonization. In these chronically infected patients, much of the bacterial burden is expected to be in the form of biofilms, which are refractory to antimicrobial therapy. However, there is a dynamic equilibrium between organisms within the biofilm and planktonic bacteria that break off and begin to replicate, behaving more like wild-type organisms with the expression of immunostimulatory exoproducts. Although our data suggest that AZM is ineffective against chronic bacteria, there may be subpopulations of bacteria within the airways that are replicating, particularly during an acute pulmonary exacerbation, and are potential targets for azithromycin. The results here presented prompt us to speculate that Azm might act not only on the host side of the infection but also to the bacteria inhibiting the release of pro-

inflammatory mediators, thus contributing to its clinical benefits recorded in CF patients. Interestingly there was a substantially greater effect of the drug on the more immunostimulatory strains, those classified as sporadic and corresponding to early host colonization events. This implies that azithromycin would have the greatest effect against early isolates of *P. aeruginosa* in CF, those that are more similar to the laboratory strain PAO1 and other environmental strains, than against the strains from patients with longstanding infection, undergoing adaptation processes to the host microenvironment. Azithromycin may also target the planktonic organisms that emerge from the bacterial biomass but may not be well-represented in sputum cultures.

Altogether the results reported indicate that:

1) the approach we describe can be of use to select potential targets to dissect the role played by the individual secreted proteins and their synergy in the pathogenesis of Pa-mediated lung disease in CF.

2) MudPIT analysis is reliable and capable in providing semi-quantitative data that can be analyzed by appropriate analytical algorithms useful for the identification of complex functional networks.

3) the analysis of CM derived from the Pa strain grown at different oxygen concentration is provided and can contribute to a better understanding of the mechanisms of survival in a microaerobic niche.

4) the differential patterns of proteins released by a clinical strain in comparison to the laboratory strain PAO1 may help in elucidating its strategy of adaptation in the CF lung as well as the pathogenesis of chronic infections. Understanding these complex mechanisms of adaptation may help in finding effective anti-bacterial drugs which target specific biomarkers responsible for the consequences of lung colonization by Pa in CF.

5) the analysis of CM derived from the Pa clinical isolates grown in absence and in presence of azithromycin is provided and can contribute to a better explaining the variable response to Pa infection and sensitivity to AZM known to occur in CF patients.

Finally, the correlation reported among MMP activity/expression and specific clinical conditions suggest that MMPs might play a role in the clinical manifestations of Pa infection supporting a link among MMP expression/activity and Pa virulence in CF patients. Future evaluation of MMP activity in a larger series of clinical isolates may provide insights on the correlation between this parameter and lung function in patients colonized by Pa strains.

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