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"Dissection of the role of *P. aeruginosa* virulence factors and host genetic background during respiratory infection "

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A. Abstract

Pseudomonas aeruginosa is the fourth most commonly isolated hospital pathogen, multidrug *re*sistant and potentially deadly causes of pneumonia. Patients at risk of acquiring *P. aeruginosa* are immunocompromised , due to immunosuppressive therapies or underlying diseases such as cancer, AIDS or the hereditary disease cystic fibrosis (CF). *P. aeruginosa*-host interaction is driven by several factors including the bacterial phenotype and the host genetic make- up.

From the bacterial side, several virulence factors have been attributed to *P. aeruginosa* pathogenicity while many others remain to be established. One third of the *P. aeruginosa* genome has no attributed homology to any previously reported sequences with only 6.7% of its genes having a function verified experimentally. To identify novel virulence genes, a genome-wide multi-host *in vitro* and *in vivo* screening was carried out. A library of 57,360 *P. aeruginosa* Tn-5 mutants was screened for phenotypic virulence traits (e.g. swarming, pyocyanin, and protease). A total of 404 Tn5-mutants showing pleiotropic phenotypes were tested in the *C. elegans* and *D. melanogaster* infection models and further reduced for validation in cell culture and animal models. In particular, 28 selected Tn5-mutants were tested for invasion and IL-8 production in immortalized respiratory A549 cells. Mortality, inflammation and lung pathology of eight selected mutants were scored in C57BL/6 mice and five out of eight mutants were significantly attenuated in inducing murine mortality. The Tn5 gene insertion sites were mapped and their conservation established across seven *P. aeruginosa* genomes by comparative sequence analysis. The Tn-5 inserted genes encoded proteins from several functional classes including hypothethical, catabolism regulation, iron uptake and quorum sensing. This screening generated a list of virulence-related genes relevant for pathogenicity in multi-hosts and favour much needed insight into the aspects of *P. aeruginosa*/host interaction.

From the host side, the clinical outcome of *P. aeruginosa* infections may be extremely variable among individuals at risk including CF patients. However, risk factors for *P. aeruginosa* infection remain largely unknown. To characterize how the genetic background influences *P. aeruginosa* lung infections, nine different inbred mouse strains were infected with *P. aeruginosa* clinical isolate and monitored for body weight change and mortality up to seven days. Next, one of the most susceptible and resistant mouse strains were further characterized by scoring bacterial count, cell-mediated immunity, cytokines and chemokines profile and lung pathology in an early time course. Susceptible mice showed a significantly higher bacterial burden, higher cytokines and chemokines levels but lower leukocyte recruitment, and a lower inflammatory severity damage when compared to resistant ones. Our results indicated that host genetic make-up may have a role in the modulation of cell-mediated immunity playing a critical role in the control of *P. aeruginosa* infection.

B. State of the Art

B.1 *Pseudomonas aeruginosa* respiratory infections: epidemiology and pathogenesis

Pseudomonas aeruginosa is an aerobic Gram negative bacterium found in several waterborne environments throughout nature [1]. It has a large genome containing a myriad of pathogenic and metabolic capabilities allowing it to infect many different organisms, including plants, amoebas, nematodes and vertebrate animals [2]. Given its ubiquitous presence in the environment and pathogenic potential, this microorganism is an important cause of both community-acquired infections (e.g. keratitis, otitis externa, skin and soft tissue infections) and hospital-acquired ones (e.g. pneumonias, urinary tract infections, surgical site and skin infections in the setting of burn injuries). Considering respiratory infection, *P. aeruginosa* can cause community acquired pneumonia (CAP) with high rate of incidence in nursing home resident, patients with chronic obstructive pulmonary disease (COPD) and patients recently discharged from the hospital. It has also been identified as the second most common cause of hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), with mortality rates of 34 to 48 %, exceeded in frequency only by *Staphilococcus aureus* [3]. In intensive care units (ICUs), *P. aeruginosa* is typically responsible for an even higher percentage of nosocomial infections, with rates of 13.2-22.6 % reported [4] [5] [6, 7].

P. aeruginosa rarely causes infections in immune-competent patients in which the acquisition of environmental bacterial cells is normally resolved by the immune system. However, *P. aeruginosa* may cause infection when normal cutaneous or mucosal barriers are broken (e.g trauma, surgery, serious burns or indwelling devices), when disruption of the protective balance of normal mucosal flora by broad spectrum antibiotic occurs, or in patients with a deficiency in the immune response caused by immunosuppressive therapies or viral infections. In particular patients at high risk of lung infections caused by *P. aeruginosa* are immunocompromised like transplant recipients, neutropenic patients and patients with HIV. In one study of patients infected with HIV, the incidence of *P. aeruginosa* bacteremia was approximately 10 times the rate of that seen in the general population of the participating hospitals, and in this category of patients *P. aeruginosa* was identified as the most common cause of bacterial bronchopneumonia [7]. Solid organ transplant and bone marrow transplant patients have increased rates of *P. aeruginosa* bacteremia, in the specific case of lung transplant the pathogen becomes an important cause of late-onset pneumonia [8].

Patients with cystic fibrosis (CF), the most frequent lethal genetic disease in Caucasian population, are particularly susceptible to *P. aeruginosa*. One of the physiological consequences of a defective CF Transmembrane Conductance Regulator (CFTR) protein in the airway epithelium is the

production of a thick and viscous mucus that creates special niches for *P. aeruginosa* colonization, leading to chronic persistence. In particular, mutations in the *cftr* gene cause a critical impairment of innate host defence systems in the lungs that results in an early and severe form of chronic airway disease featuring mucus obstruction, neutrophil dominated airway inflammation and bacterial infection, finally leading to progressive pulmonary damage with bronchiectasis and emphysema[9] [10] [11]. Early respiratory insufficiency due to chronic airway inflammation with recurrent *P. aeruginosa* infection is considered the major cause of morbidity and mortality in CF patients. About 80 % of adult CF patients suffer from chronic *P. aeruginosa* lung infections which are responsible for their short life expectancy with a median predicted survival of 37.8 years (**Fig.1**) [12].

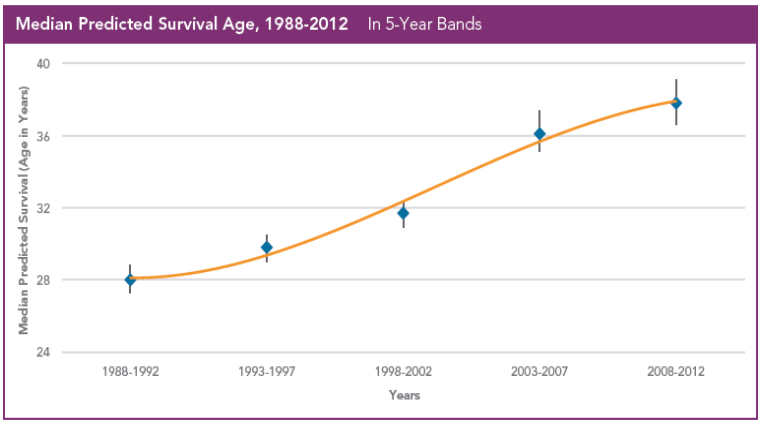

Figure 1: Median predicted survival age in CF patients. Rise in median predicted survival age since 1988 in 5-year bands or groups. For 2008-2012, the median predicted survival was 37.8 years.[12].

B.2 Pathogenesis of cystic fibrosis lung disease

B.2.1 Epidemiology of CF: CFTR structure, function and mutations

CF, cause by mutations in the *cftr* gene, is the most common life shortening, autosomal, recessive monogenic disease which affects approximately 30.000 individuals in the United States and about 70.000 worldwide.

The 230 kb *cftr* gene, with 27 exons located at the long arm of chromosome 7 (7q31.3), encodes a c-AMP responsive chloride channel in lipid bilayers of the apical membrane of epithelial cells lining the respiratory and digestive tracts. The CFTR is the only known member of the ABC family that acts as an ion channel [13, 14]. The mature 1480-residue long membrane protein is composed by two transmembrane domains, two nucleotide domains, an additional regulatory region, as well as N- and C-terminal extensions of about 80 and 30 residues in length, respectively. Each membrane-spanning domain contains six membrane-spanning alpha helixes portions forming a chloride-conductance pore. Channel activity is governed by the two nucleotide binding domains and the

regulatory domain, while the C-terminal domain, that is anchored to the cytoskeleton, interacts with other proteins that influence CFTR function (**Fig.2**).

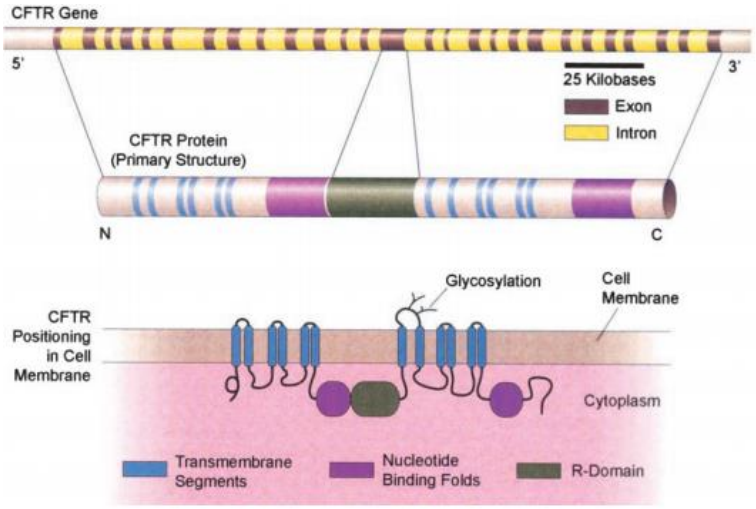

Figure 2: CF transmembrane conductance regulator (*cftr*) gene and its encoded polypeptide

Although CFTR is primarily a chloride channel, it also control the transport of other ions and molecules as well as the function of several membrane channels, such as the epithelial sodium channel (ENaC), potassium channels and outwardly rectifying chloride channel [15-17]. Consequently CFTR regulates the hydration of the airways' surface fluid.

To date, there are more than 1900 different mutations in the *cftr* gene that are known to cause CF, but over than 90 % of cases are associated with a recessive deletion mutation of phenylalanine at position 508 (ΔF 508) [18]. CFTR mutations are classified into 5 groups: Class 1 mutations cause a defect in CFTR protein synthesis , such as the premature stop codon W128X; Class 2 mutations, including the first most common ΔF508, are translated into full length nascent polypeptide chains, but are defective in folding and targeted for degradation rather than trafficked to plasma membrane; Class 3 CFTR mutants are able to reach the plasma membrane but have channel gating defects that decrease channel opening time and decrease chloride flux, e.g the second most common mutation G551D; Class 4 mutants reach the plasma membrane but have decreased channel conductance even when the gate is open; and Class 5 mutants show a fully functional CFTR at the level of plasma membrane with reduced abundance due to defective mRNA splicing. Each of these classes of mutations cause a multi organ disorder in which the level of functional CFTR is important in determining the severity of disease.

B.2.2. Impact of defective CFTR on airway physiology and mucociliary clearance

In the lung, CFTR conducts $HCO3^-$ and Cl^- thereby helping to regulate the airway surface liquid (ASL) volume and composition also by interacting with other channel such as the ENaC. In the respiratory tract, ASL, including the periciliary liquid layer (PCL) and the mucus layer, is a first line of defence against inhaled or aspirated pathogens providing a barrier between the epithelium and inspired air and its regulation reflects the balance between Na^+ absorption and Cl^- secretion mediated by EnaC and CFTR channels respectively. The PCL covers the cilia, hydrating mucins and allowing for ciliary beating by distancing the mucus from the cell surface. The mucus layer is comprised of secreted and tethered mucins produced by surface goblet cells and submucosal gland epithelia (**Fig.3**).

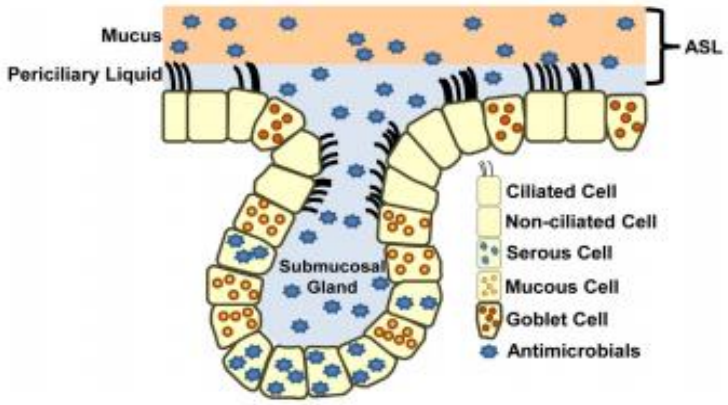

Figure 3: A simplified model of the airway epithelium. The airway epithelium has two compartments, the surface epithelium and the submucosal gland (SMG) epithelium. The airway surface epithelium includes ciliated and non-ciliated cells, goblet cells. SMG epithelium includes ciliated duct cells, mucus cells and serous cells that secret antimicrobial proteins. Airways surface liquid (ASL) is composed of two layers a mucus gel layer and a periciliary layer.

In a normal epithelium the presence of an hydrated and low viscous PCL promotes an efficient mucocilliary clearance of pathogen trapped in the mucus layer. A normal rate of epithelial cells oxygen consumption results in homogenous pressure of oxygen (pO_2) within the ASL (**Fig.4a**). On the contrary in CF airways, the absence of a functional CFTR induces a hyper absorption of Na^+ and water across the epithelia. This phenomenon generates a volume depletion of the PCL, resulting in reduced mucocilliary clearance (**Fig.4b**). A persistent mucus hypersecretion by secretory gland/goblet cells increases the height of the luminal mucus layer/plugs (**Fig.4c**). In addition the accelerated Na^+ absorption is fuelled by an increased turnover rate of ATP- consuming Na-K-ATPase pumps leading to two-to threefold increases in CF airway epithelial O_2 consumption, contributing to create a stepper O_2 gradient in the ASL.

Moreover an impaired $HCO3^-$ secretion leads to a decrease of ASL pH. Of note, mucins become more viscous at acidic pH and this may contribute to the thick hypoxic mucus plaque formation that characterize CF airways as an expression of the persistent and uncontrolled hyper secretory activity

of mucus gland. It was also well described that changes in pH of CF ASL negatively impact on the activity of many antimicrobials. The cathelicidin LL37, for example, undergoes a conformation change that decreases its antibacterial activity [19]. An acidic environment also reduces the amount of human β defensin1 produced by airway epithelial cells [20].The pH sensitivity of numerous airway antimicrobials is a probable causal link between reduced ASL pH in CF and the impaired antibacterial activity of CF ASL, and consequent bacterial infections.

The hypoxic gradient in the thickened mucus layer creates a favourable environment for the opportunistic *P. aeruginosa*, able to penetrate actively and/or passively due to mucus turbulence into hypoxic zones within mucus masses. In these conditions the bacterium adapts to hypoxic niches with increased alginate production and macro colonies creation (**Fig.4d,e**). These macro-colonies are resistant to secondary defences including neutrophils and antibiotic treatment (**Fig.4f**). All above-mentioned pathogenic events favour the persistence of *P. aeruginosa*, setting the stage for the establishment of chronic infection. [21]

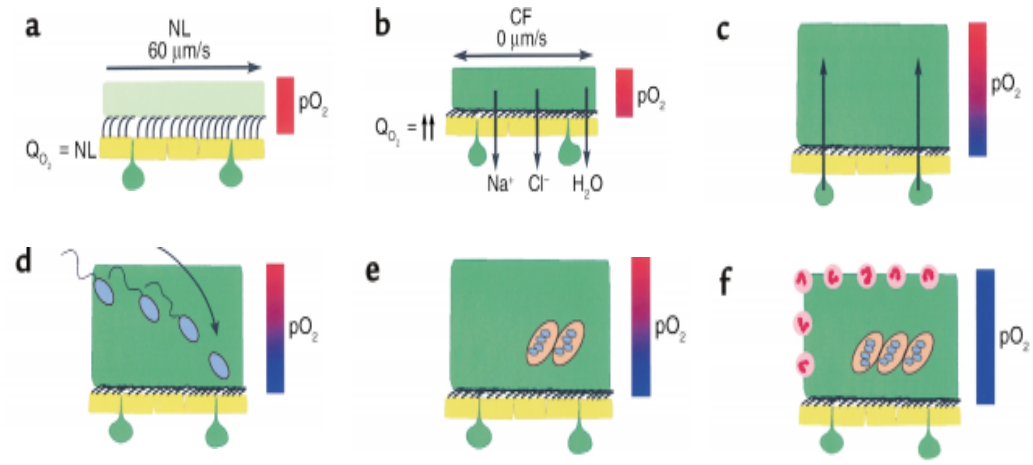

Figure 4: **Schematic view of the pathogenic events that leads *P. aeruginosa* to establish chronic infection in airways of CF patients [21]**

B.2.3 Microbiology of CF lung disease

Traditional aerobic culture-based studies of spontaneous expectorated sputa indicate that CF airways infection involves a relatively small collection of pathogens that are typically acquired in an age- dependent sequence, beginning early in life with *Haemophilus influenzae* followed by *S. aureus* and culminating in chronic infections dominated by *P. aeruginosa* or *Burkolderia cepacia* complex species (**Fig.5**). Additional opportunistic pathogens including *Stenotrophomonas maltophila*, *Achromobacter xyloxidans* and nontubercolous mycobacterium have been recovered from adult patients with increasing frequency.

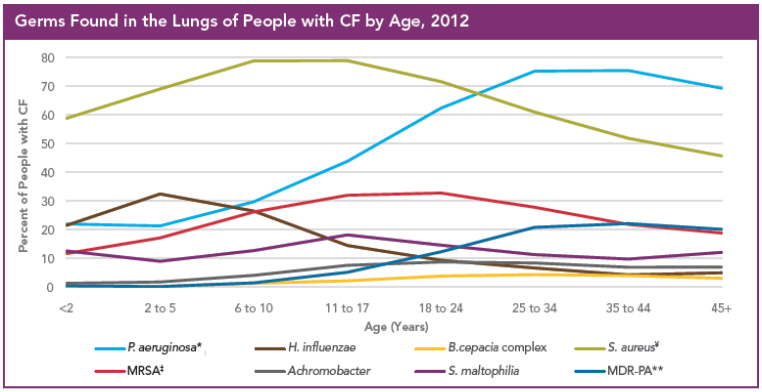

Figure 5: Prevalences of several common respiratory pathogens in CF as a function of age. [12]

Studies on CF respiratory specimens analysed under strict anaerobic culture conditions show that anaerobic bacterial species are also present within CF airways in high numbers. The spectrum of facultative and obligate anaerobic species includes members of the genera *Streptococcus*, *Prevotella*, *Actinomyces* and *Veilonella*.

P. aeruginosa is by far the most significant pathogen in CF. Based on immune response in young children, infection appears to occur much earlier than believed previously, in fact the mean age of detection of an antibody response to *P. aeruginosa* is 15 months, whereas the mean age of the first positive airway culture is 23 months [22] [23]. Risk factors for initial *P. aeruginosa* airway infection in patients with CF diagnosed by new born screening included female sex, homozygous ΔF508 genotype, and *S. aureus* precolonization. Up to 80% of patients with CF are eventually infected with this organism, and its acquisition is associated to lung tissue deterioration. In particular in one longitudinal study that combined the culture of respiratory samples with serological infection, more than 97.5 % of CF patients were found to be infected with *P. aeruginosa* by the age of three years. The source of *P. aeruginosa* isolates in patients has not been clearly established even if some patients appear to have acquired infection from social contacts or hospital settings, the source of initial acquisition seems to be environmental reservoirs. Thereafter, the colonizing bacterial strains persist in the airways of CF patients for several years by adapting their phenotype to the host conditions. Paediatric CF patients with respiratory cultures positive for *P. aeruginosa* experience higher mortality (8 years risk of death was 2.6 times higher), an increased frequency of infection and hospitalisation for acute respiratory exacerbations, and a decreased lung function with a significantly lower percent of predicted forced expiratory volume in 1 sec (FEV_1) when compared to those without *P. aeruginosa* [24]. Repeated cycles of infection and inflammation during post-natal period result in structural abnormalities in CF patients despite essentially normal

architecture of the lungs at birth. With established infection, formation of micro-abscesses leads to collapse of the distal airways. The reduced ventilation and consequent hypoxia result in airway remodelling. This process is considered an important cause of irreversible airway narrowing and consequent constitutive airflow limitations in a lung strictly damaged. Pathologic characteristics of CF lung disease include squamous metaplasia, submucosa mucus gland enlargement, increase of airway smooth muscle mass, and reticular basement membrane thickening highlighted by deposition of tenascin and the fibrillar collagens I and III in the sub-epithelial bronchial tissue matrix[25-27]Finally, pulmonary insufficiency, mainly due to *P. aeruginosa* chronic infection, is responsible for at least 80 % of CF-related deaths [12].

S. aureus is the bacterial pathogen most frequently cultured in the paediatric CF population and remains an important pathogen throughout adulthood. The CF community is particularly concerned about methicillin-resistant *S. aureus* (MRSA). Some data suggest slightly worse clinical outcomes in patients with chronic MRSA infections than in those with methicillin- sensitive *S. aureus* infections, because of greater airflow obstruction, increased hospital admissions and extended courses of antibiotics [27]. In addition *S. aureus* might have a negative effect on post-transplantation outcomes. Actually it was associated with an increased long term mortality and more frequent episodes of acute cellular rejection within the first 90 days of transplantation. Similarly another retrospective study showed that *de-novo* acquisition of MRSA after transplantation resulted in increased mortality compared with transplant patients without MRSA infections [28].

Atypical mycobacteria are sometimes found in airway secretions from CF patients; it remains unclear whether this finding represents true infection in all cases, or is only saprophagous colonisation in some patients. *Mycobacterium avium* complex (72%) and *Mycobacterium abscessus* (16%) were the most common atypical mycobacteria found in a survey of US cystic fibrosis centres[29].

Other pathogens like *S. maltophila* or *A. xyloxidans*, may be present but generally carry no worse prognosis. The prognosis dramatically becomes worst after the acquisition of *B. cepacia* complex that cause a sepsis-like syndrome characterized by high fever, bacteremia and a rapid progression to severe necrotizing pneumonia leading to death. Recent studies on fungal colonization/infection of the CF airway show the presence of *Aspergillus fumigatus* in up to 50% of subjects and other moulds together with yeasts such as *Candida*, isolated from almost 50 to 75% of CF patients. In particular the isolation of *A. fumigatus* from the airways of children with CF is associated with a lower forced expiratory volume in 1 second (FEV_1) and an increased risk of pulmonary exacerbations requiring hospitalization [30]. Fungal colonization and infection of CF airways late in

disease progression is very common and not so surprising considering the exposure to frequent broad spectrum antibiotic therapy [31]. In particular Candida colonization was associated with worse clinical status (e.g. osteopenia, pancreatic insufficiency) and also with co-colonization with *P. aeruginosa* and *A. fumigatus.*

B.3 *P. aeruginosa* genome

The first completed *P. aeruginosa* genome sequencing was performed for strain PAO1 [32], derived from an Australian wound isolate in 1950s. PAO1 strain is still the major reference strain for genetic and functional studies on *P. aeruginosa*.

PAO1 genome consists of a 6.264-Mbp circular chromosome encoding 5,570 predicted protein coding sequences. It also carries a lot of chromosome-mobilizing plasmids that are very significant to the organism 'lifestyle as a pathogen. The plasmids TEM, OXA and PSE, for instance, encode for β-lactamase production, which is necessary for its resistance to antibiotics. The size of the PAO1 genome is the result of genetic complexity (more genes of unique function) rather than gene duplication. *P. aeruginosa* genome has indeed a large number of paralogous groups whose members encode distinct genes functions. The significantly higher percentage of these genes groups found in *P. aeruginosa* genome when compared to those present in other bacterial genomes, indicates that environmental versatility has favoured a process of expansion of genetic capability of *P. aeruginosa* genome rather than gene duplication.

Notably there is still one third of *P. aeruginosa* genome with no attributed homology to any previously reported sequences. In detail, only the 6.7 % of *P. aeruginosa* genome (372 ORFs) are known genes with demonstrated functions (confidence level 1) primarily encoding lipopolysaccharide biosynthetic enzymes, virulence factors, such as exoenzymes and their secretory system, and protein involved in motility and adhesion. The 19 % of *P. aeruginosa* genome (1,059 ORFs) are genes with strong homology to those found in other organism with demonstrated functions (confidence level 2) including those required for DNA replication, protein synthesis, cell-wall biosynthesis and intermediary metabolism. The 28.5 % of *P. aeruginosa* genome (1,590 ORFs) are genes with assigned probable function on the basis of similarity to established sequence motif (confidence level 3) that belongs to functional classes including putative enzymes (405 genes), transcriptional regulators (341 genes), or transporters of small molecules (408 genes). Finally a large proportion of the genome (45.8% of ORFs) consists of genes for which no function could be determined or proposed (confidence level 4). Of these nearly a third (769 ORFs) shares homology to genes with unknown function predicted in other bacterial genomes and the remainder 32% of ORFs does not have strong homology with any reported sequence [32]. Consequently there is still a

lot of information to be uncovered with regards to the mechanism used by these organism to cause disease, with an array of novel virulence factors remaining to be discovered within its genome.

In addition to PAO1, other 14 *P. aeruginosa* strain genome are available as completed genomes or listed as being draft assemblies or incomplete. The second *P. aeruginosa* genome sequence was published for the strain PA14 [33] a clinical strain more virulent than PAO1, first isolated from an human burn patient. Among the other strains whose genome has been sequenced the "Liverpool epidemic strain" LESB58 was found to be highly transmissible among CF patients causing severe infection even in non CF human hosts [34, 35]. PA7 belongs to a collection of 10 non-respiratory clinical isolates (3 from Thailand; 7 including PA7 from the Malbran Institute in Buenos Aires, Argentina) that were collected for their unusual resistance patterns sharing only 93.5 % nucleotide identity in the core genome with the other sequenced strains [36]. Almost complete genome sequences are also available for strains 2192, C3719, PACS2 and 39016 (www.pseudomonasaeruginosa.com).

P. aeruginosa has the highest proportion of predicted regulatory genes observed in the sequenced bacterial genomes corresponding to the 8.4%. These genes, in addition to the remarkable gene complexity, enable *P. aeruginosa* to evolutionary adapt itself to different environmental conditions and hosts. Moreover the bacterium has broad capabilities to transport, metabolize and grow on organic substances, numerous iron-siderophore uptake systems and enhanced ability to export compounds (enzymes and antibiotics) by a large number of protein secretion and RND efflux systems contributing to its intrinsic resistance. Five different classes of secretion systems have been described which have been identified as type I secretion system (T1SS) up to type VI secretion system (T6SS) [37], with the exception of TIVSS, as well as a number of genes for chemotaxis (four chemotaxis systems) and motility [32].

P. aeruginosa genome is composed by a conserved core component defined as comprising all genes present in all sequenced genomes of *P. aeruginosa* and an accessory component consisting of additional strain specific regions. The accessory genome consists of extra-chromosomal elements like plasmids and blocks of DNA inserted into the chromosome at various loci. The elements of the accessory genome were acquired by horizontal gene transfer from different sources including other species or genera. Therefore the *P. aeruginosa* genome is often described as a mosaic structure of conserved core genome frequently interrupted by inserted parts of the accessory genome. This mosaic also shows remarkable plasticity. Actually, events of acquisition of new foreign DNA as well as larger or smaller deletion events, single nucleotide mutations and even chromosomal inversions, potentially affecting parts of the core and/or accessory genome, continuously modify the genome. Within the chromosomally integrated islands, very often phages, transposons or IS-

elements are found. Many elements were irreversibly fixed by secondary mutations or deletions, but a few others have retained their mobility and can still leave the chromosomal insertion site and be transferred elsewhere, as shown for the elements PAPI-1 and pKLC102 [38]. Among the genomic islands of the *P. aeruginosa* accessory genome, members of the PKLC102 family are highly prevalent. They represent a special group of ICEs (Integrative and Conjugative Elements) that generally consist of individual semi-conserved DNA blocks. This conserved gene set accounts for structural and mobility-related features and conjugal transfer. Individual genes within the islands can encode a broad spectrum of different functions, among them catabolic pathways as well as virulence effectors.

A comprehensive comparison of the genomes of *P. aeruginosa* strains PAO1, PA14, 2192, C3719, and PACS2 [39] led to the definition of so called "regions of genome plasticity" (RGP) that are polymorphic strain-specific segments dispersed in the circular chromosome and flanked by conserved genes referred to as anchors (**Fig.6**). These RGP include any region of at least four contiguous ORFs that are missing in at least one of the genomes explored. Mathee and co-workers in 2008 analysed the genome sequences of three *P. aeruginosa* strains isolated from CF patients (PA2192, PACS2 and C3719). PA2192 exhibits major phenotypic adaptation including conversion to mucoidy (due to a non-sense mutation in its *muc*A gene), while C3719, a representative of the Manchester Epidemic Strain, is known for its enhanced virulence and transmissibility. Comparison of the annotated genes in the *P. aeruginosa* PA2192 and C3719 genomes with those in the genome of PAO1, PA14 and PACS2, revealed an extensive conservation of a set of genes that are shared by all of the strains. There are 5,021 genes that are conserved across all five genomes analysed, with at least 70% of sequence identity (**Fig.6**). In addition, each sequenced *P. aeruginosa* strain carries about 40 RGPs with insertions. The RGPs can be sites of insertions of common or unique genomic islands and bacteriophage genomes, or the result of deletions of particular segments of DNA in one or more strains. The islands inserted at RGPs often differ among genomes indicating the individual evolutionary trajectories of the strains, whilst also maintaining core virulence functions.

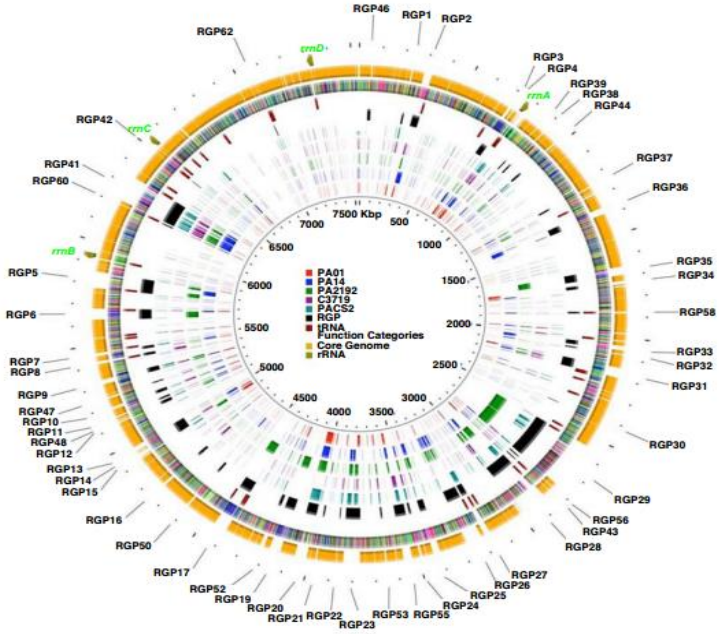

Figure 6: The *P. aeruginosa* pangenome. Using the core genes from PA14 as the template, all of the accessory genes from PA2192, C3719, PAO1 and PACS2 were integrated. The outer circle (gold) indicates the core genes, the second circle shows the functional annotations. The third circle indicates the position of t RNAs. The accessory genes in the inner circles are from PA14 (blue), PA2192(green), C3719 (purple), PAO1 (red), and PACS2 (teal). The outer green arrows show the positions of rRNAs. [39]

B.3.1 A different repertoire of *P. aeruginosa* virulence factors during acute and chronic stage of infection

Central to the success of *P. aeruginosa* infection is the genetic flexibility provided by its large genome and its ability to adapt to the pressure imposed by the host mucosal immunity. Actually the pathogenesis of *P. aeruginosa* pneumonia is complex, and the outcome of an infection depends on the virulence factors displayed by the bacteria, as well as the host response. Planktonic organisms aspirated from the environment up- regulate a specific set of genes involved in motility, proteolytic activity and carbon utilization. Later, adaptation results at least in part from the selection of clonal lineages containing spontaneously arising mutations. In particular several mechanisms of differential gene expression, gene mutation, extensive genomic rearrangements and acquisition of large blocks of DNA by co-infecting bacterial species are responsible for generating pathogenic variants from the acquired strains.

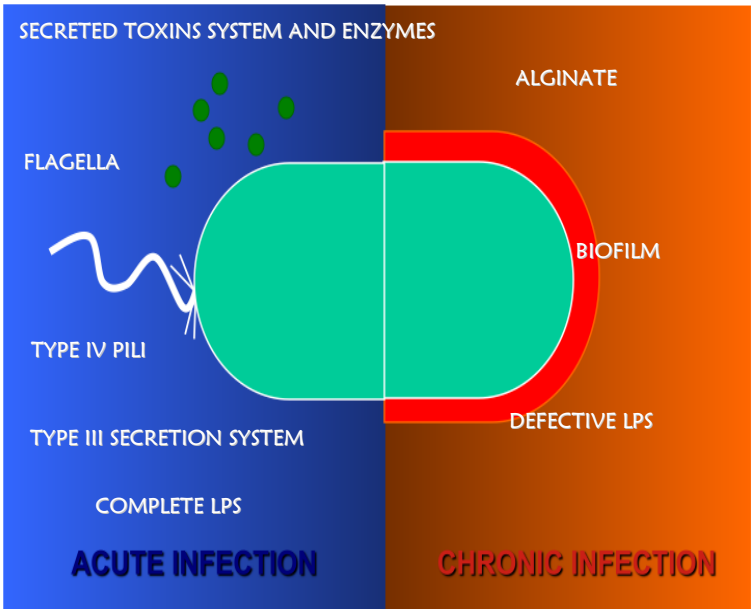

Figure 7: *P. aeruginosa* virulence factors during acute and chronic stage of infection. It has been described that *Pseudomonas aeruginosa* during the acute phase of infection is characterized by a specific arsenal of virulence factors like flagella, type IV pili, type III secretion system, secreted toxins system and enzymes and complete LPS. Next the long term colonization of CF airways selects pathoadaptive variants of the pathogen with virulence factors different from the initially acquired ones (a defective LPS, alginate and biofilm production)

During the initial colonization phase a potent array of *P. aeruginosa* extracellular and cell bound virulence factors are critical for the acute phase of infection and then invasion, settlement and dissemination throughout the host organism (**Fig.7**). Among the several surface structures for motility and adhesion in biotic and abiotic environments, there are a single polar flagellum, polar type IV pili and chaperone/usher pili (cup) fimbriae. Flagella and pili, the motile surface appendages of *P. aeruginosa*, are responsible for bacterial motility, progression towards epithelial contact and dissemination throughout the host organism. In particular flagella is considered one of the most important virulence factors in acute infection. These complex proteic structures form a motile filamentous polar appendage at the surface allowing the swimming movement of *P. aeruginosa*. Interaction with epithelial cells through binding to Toll-like receptors TLR2 and TLR5 triggers the NFkB inflammatory pathway through activation of the extracellular regulated kinase pathway, leading to IL-8 production [40]. Pili or fimbriae are smaller filamentous surface appendages involved in twitching motility allowing *P. aeruginosa* to spread along hydrated surfaces rather than swim. This feature facilitates the rapid colonization of the airway, playing a pivotal role for the adhesion phase of colonization through the binding to asialoGM component of the epithelial cell membrane [40].

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. It associates a hydrophobic domain, Lipid A, inserted into the phospholipid bilayer, to a hydrophilic tail composed of the core polysaccharide and the O-specific polysaccharide. The

variable O-specific polysaccharide chains are the basis of antigenic identification of *P. aeruginosa* serotypes. Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors proteins MyD88 and TRIF which mediate the activation respectively of proinflammatory cytokines and Type I interferon gene (IFNγ) [41].

A major virulence factor associated with increased *P. aeruginosa* aggressiveness is the type III secretion system (T3SS). The T3SS allows *P. aeruginosa* to inject secreted toxins through a syringe-like apparatus, directly into the eukaryotic cytoplasm. Several of the translocated T3SS effector proteins can modulate innate immune recognition of bacteria or target effector mechanisms of the innate immune system. These include Exoenzyme U (ExoU), a phospholipase A_2 that can inhibit inflammosome activation by *P. aeruginosa* and that causes rapid necrotic cell death [42]. Exoenzymes S (ExoS) and T (ExoT) are enzymes able to inhibit migration and phagocytosis of macrophages and neutrophils [43] [44]. Exo Y is an adenylate cyclase that increases intracellular levels of cAMP.

Other virulence factors secreted via T2SS into the extracellular space such as elastase, alkaline protease, exotoxin A, and phospholipase C are also liable for invasion and dissemination by destroying the protective glycocalix of the respiratory epithelium and exposing epithelial ligands to *P. aeruginosa* and participate in cytotoxicity [45]. Among the alkaline protease secreted, AprA is an important virulence factor involved in specific strategies of immune escaping by degradation of the central complement protein C3, IFN-gamma and monomeric form of flagellin.

In addition, *P. aeruginosa* produces a number of small molecules that are directly inhibitory or toxic to immune cells. Pyocyanin a redox-active phenazine can trigger neutrophil apoptosis and it may also affect CFTR function by blocking Cl^- transport in human bronchial cells [46]. Bacteria that cannot produce this molecule are cleared more efficiently from the acutely infected mouse lung than isogenic wild type organism [47]. Pyocyanin is associated with disease severity and decline of lung function and it contributes to the dominance of *P. aeruginosa* in the CF lung.

Pyoverdine is a yellow-green fluorescent siderophore, a small molecule chelating iron from the environment for use in *P. aeruginosa* metabolism. The rhamnolipids, other secreted small molecules, cause neutrophil necrosis. It was described that bacterial mutants deficient in their synthesis are more rapidly cleared from mouse lungs in a model of agar beads chronic infection [48]. In addition rhamnolipid production is positively regulated by signals of the quorum sensing (QS), a cell density monitoring mechanism, and their expression is greater when bacteria are in stationary phase or high-density growth conditions, such as biofilms. However, their relative importance in acute *P. aeruginosa* infections appears to be less than that of the T3SS or T2SS substrates discussed before [49]

During the transition from acute to chronic infection several *P. aeruginosa* factors that exert destructive and/or immunostimolatory effects are reduced and most of *P. aeruginosa* invasive functions are selected against in CF chronic infection leading to more persistent phenotypes [50] [51, 52](**Fig. 7**). It has been described that the chronic stage of lung infection is frequently associated with a dramatic diversification of the *P. aeruginosa* persisting clone into various morphotypes or niche specialist, a process known as adaptive radiation [53]. These pathoadaptive variants are indeed characterized by alterations in *P. aeruginosa* structures, with respect to the early acquired strains. These phenotypic changes may represent an "immune evasion strategy", necessary for long-term colonization of the CF host mediated by *P. aeruginosa* pathoadaptive lineages.

In addition, during advanced stage of infection there is an increased expression of genes required for survival within the nitrogen-rich, nutrient-deficient CF lung [54] [55] and pathways involved in iron scavenging.

P. aeruginosa is capable of adapting to CF airways by selecting pathoadaptive variants without flagella and pili in order to evade immune surveillance and chronically persist in CF patients [56]. The loss of engagement by phagocytic receptors, that recognize flagellar components, and the loss of immune activation, through flagellin-mediated TLR signalling, indeed reduce host immune response. Amiel and colleagues described that loss of *P. aeruginosa* motility dramatically alters immune responses to these bacteria compared to those for motile isogenic bacterial strains and that it is the loss of flagellum-mediated motility, but not flagellum expression itself, that results in dramatic bacterial resistance to phagocytosis by murine and human phagocytes [57]. Likewise, studies in the agar beads murine model by using the *P. aeruginosa* isolates from patients with CF demonstrated that the risk of chronic infection is increased by the absence of pili and flagella [51]. These studies provide an explanation for the clinical observation that *P. aeruginosa* isolates obtained from CF hosts often exhibit a non-motile phenotype [58] and indicate how this phenotype can confer a survival advantage during chronic infection. In addition *P. aeruginosa* down-regulates the expression of several cytotoxic factors over the course of infection to establish a long-term presence in CF airways, like for example pyocyanin and T3SS. Modification of LPS appears to be one of the main factors in the adaptation of *P. aeruginosa* during chronic CF infection. It is already known that *P. aeruginosa* consistently changes LPS lipid A structure in the evolution from acute to chronic infections. Generally production of fully hexa-acylated lipid A is associated with a more strong TLR4 mediated inflammatory response while lipid A with lower levels of acylation triggers reduced cellular responses [59]. Actually lipid A component isolated from clinical strains of *P. aeruginosa* often results in a blend of different species with a biological activity that might be different when compared to single species bioactivity [60]. In 2009 Cigana and co-workers

determined the LPS structures of three *P. aeruginosa* clones isolated from airways of a CF patient during a period of 7.5 years. Lipid A acylation was temporally associated with different stages of CF infection. Among the three strains, LPS lipid A diversity was observed in the number and position of fatty-acid side chains, in particular a predominance of highly acylated LPS structures was observed in *P. aeruginosa* pathoadaptive variants, isolated after years of colonization. Lipid A moieties of late adapted strains displayed a reduced immune-modulatory TLR4-mediated activity compared to LPS of the clonal strain isolated at the onset of infection. In particular, a weaker NF-kB and IL-8 inflammatory response *in vitro*, and reduced neutrophils recruitment and cytokines levels were observed *in vivo* after stimulation with lipid A from late strains with respect to lipid A of early strains emphasizing the reduced immune-potential of LPS of late colonizer *P. aeruginosa* strains [60]. Modification of pathogen associated molecular patterns (PAMPs), such as lipid A, but also peptidoglycan muropeptides [60], could thus hijack genes involved in innate response favouring *P. aeruginosa* survival.

An indication of chronic stage of *P. aeruginosa* infection is the predominance of mucoid strains representing the end result of failed innate immunity and an effective bacterial adaptation to the host. Mucoid *P. aeruginosa* is characterized by the over production of alginate, that is an exopolysaccharide made of repeating polymers of mannuronic and glucuronic acid. Alginate over-production has been linked to mutations in a gene cluster designated as the *mucABCD*, in particular *mucA* mutants have been isolated from chronically infected CF patients [61]. Surrounding conditions in CF patients lungs and host inflammatory response increase alginate synthesis leading to conversion to an alginate overproducing mucoid phenotype [62]. Overexpressed alginate protects *P. aeruginosa* against reactive oxygen species and antibiotics [63] [64] [65] and from the host defence including scavenging of free radicals released by macrophages, providing a physical barrier that impairs phagocytosis, inhibiting neutrophil chemotaxis and complement activation [66]. The response of airway epithelia to the stimuli presented by mucoid *P. aeruginosa* is not pro-inflammatory and, hence, may not be conducive to the effective elimination of the pathogen [67]. Indeed, *in vivo* studies suggest that clearance of mucoid strains from murine lungs is diminished compared with non mucoid strains, indicating improved survival of alginate-producing strains in the respiratory tract [68] [69].

Once a critical mass of bacteria is present in the airways *in vivo* data indicate that they form a cluster of micro-colonies that are encased in a biopolymer matrix called biofilm [70] [71], widely diffuse during chronic infection. *P. aeruginosa* biofilms are believed to arise in the respiratory tract of CF patients through a series of steps beginning with the attachment of planktonic (i.e free swimming) *P. aeruginosa* to epithelial cells or debris within the airway [66]. In the process of

biofilm formation, colonies of *P. aeruginosa* will secrete exopolysaccharides, including alginate, resulting in the production of a matrix characterized by a complex architecture of bacterial micro-colonies. In this microbial community the expression of several virulence factors is coordinated by the QS system. This mechanism allows bacteria-to-bacteria cell signalling through highly soluble quorum sensors, such as the *Pseudomonas* homoserine lactone and quinolones, which act in concert with specific transcriptional activators. The *P. aeruginosa* QS system consists of 2 *N*-acyl-homoserine lactone (AHL) regulatory circuits (*las* and *rhl*) linked to an alkyl-quinolone (AQ) QS system. LasR and RhlR are both LuxR transcriptional activators, which activity depends on AHLs synthesized via LasI and RhlI, respectively, to drive virulence gene expression. In various infection models, mutants defective in QS are significantly less pathogenic than their parental strains suggesting that QS systems may be an interesting target for the prevention of acute and chronic *P. aeruginosa* infections [72]. The adaptation process might also be supported by the emergence of hypermutable strains characterized by an increased random spontaneous mutation rate, due to defect in genes *mutS*, *mutL* and *uvrD* involved in DNA repair or error avoidance systems. In a first work published by Oliver et al in 2000, an extremely high prevalence (20% of isolates, 37% of patients) of mutator *P. aeruginosa* was found in chronically colonized CF patients [73]. Regarding the genetic basis of hypermutation in *P. aeruginosa* strains from CF patients, by far the most widely investigated mechanism is the MMR system that detects and repairs replication errors including any kind of mispairs and short insertions or deletions. Up to 60-90% of the mutator isolates from CF have a defective MMR system, mainly caused by mutation of *mutS* or *mutL*. Most important, *P. aeruginosa* hypermutability, which was previously associated with an important biological cost [74], turns to be a benefit under selection pressure, such as antibiotic treatments, in a context of CF chronic airways infection, and can contribute to lung damage during long-term persistence [75], becoming a relevant problem in the management of chronic infections.

All these findings suggest that immune escape strategies, like mucoid conversion, biofilm growth, PAMPs modifications and inhibition of invasive virulence determinants, may confer a selective advantage for the establishment of *P. aeruginosa* chronic colonization and persistence.

B.4 Influence of host genetic make-up influences the outcome of infection diseases

As shown by studies of genetic susceptibility to infections in human disease, the complexity of host-microbe interaction depends on the pathogen with its unique repertoire of virulence factors, but also on the host with its specific genetic background. Some of the clearest manifestations of this genetic component include the well-known examples of malaria resistance in individuals

heterozygous for haemoglobinopathy alleles (HbS) and human immunodeficiency virus (HIV) resistance in individuals carrying variance of the viral co-receptor chemokine receptor 5 (CCR5) [76] [77]. It has also been described that particular individuals with polymorphic variants of natural resistance-associated macrophage protein 1 (Nramp1) are susceptible to mycobacterial infections and that humans harbouring mutations in genes regulating phagocytes activation (IFNGR1, IFNGR2, IL12B, IL12RB1 and STAT1) are susceptible to recurrent typhoidal *Salmonella* infection [78] . In addition, *TLR4* gene polymorphisms are associated with susceptibility to various infectious and non-infectious disease. Two described polymorphism, Asp299Gly and Thr399Ile, were originally associated with the lung response to inhaled LPS in healthy human volunteers [79]. These polymorphism belonging to a specific genetic background, predispose to an increased risk of Gram–negative infection [80], sepsis [81], severe inflammatory response syndrome [82], severe malaria [83], brucellosis [84], and respiratory syncytial virus disease [85].

Study of infrequent single-gene effects in humans is useful for better understanding the molecular pathogenesis of infection revealing also host defence mechanisms that can potentially be used as novel targets for therapeutic intervention. However linkage and association studies show that the genetic component of susceptibility to infection is usually complex and multigenic, reflecting the plurality of cell types and biochemical pathways involved in both the initial sensing and the dynamic response to a pathogen. An evident example of complex disease is CF. Although it is recognized as a monogenic disorder, a considerable heterogeneity in its clinical outcome has been documented since the description of the disease. Studies on twins and sibs models have determined that non CFTR-genetic factors account for more than 50% of variability in lung disease [86]. The hypothesis that variation in phenotype can be explained by allelic variants has been put forward, and many studies have attempted to link the type of CFTR mutations to the severity of the disease. Good genotype–phenotype correlations have been reported for pancreatic enzyme function [87, 88]. However, analysis of the effects of CFTR mutations on pulmonary function, which represents the major cause of morbidity and mortality in CF, has failed to establish a strong genotype–phenotype correlation [89] and it remains enigmatic how these mutations cause enhanced susceptibility to pulmonary infection and how this susceptibility might be prevented [90]. Furthermore there is a remarkable heterogeneity among CF patients, in the time of onset and severity of chronic bronco pulmonary *P. aeruginosa* infection [91] even within those carrying the same *cftr* genotype. Identification of additional gene alleles that directly influence the phenotype of CF disease became a challenge in the late ‘90ies, not only for the insight it provides into the CF pathophysiology, but also for the development of new potential therapeutic targets [89].

Two genetic approaches have mainly been explored, the candidate gene approach and the wide genome association (GWA) approach. In the candidate gene approach the genes investigated are selected from the current understanding of CF lung disease pathophysiology with a special interest in factors involved in the inflammatory and infectious response, the ion transports and the cytoskeleton. Some examples of most relevant findings show that polymorphic variants of *TGFB1* have been involved in various respiratory diseases such as pulmonary fibrosis, asthma, COPD, and also associated with more severe CF lung disease [92], with some controversial findings maybe correlated to population ancestry and environmental factors [93]. Also three polymorphism found in *IL8* gene were associated with CF pulmonary disease severity [94]. MBL2 is an innate immune protein accumulated in the lung during acute inflammation that plays a major role in infectious disorders binding several bacteria including *P. aeruginosa* and *S. aureus*. Chalmers and colleagues correlate *MBL2* deficiency alleles to an earlier *P. aeruginosa* acquisition, reduced pulmonary function among adults and increased rate of death or requirement of lung transplantation. Polymorphism located in *TLR4* and *TLR5* genes instead did not show significant association with lung function or age at first *P. aeruginosa* acquisition in CF young patients [95].

In order to overcome the limitations linked to small sample size and poor study design of the candidate gene approach, linkage analysis program and GWA studies were set up. The wide genome approach includes indeed linkage analysis and association analysis, both based on polymorphism linked to the gene responsible for disease susceptibility. By linkage analysis, familial segregation of disease can be followed through the phenotype and linkage can be established between phenotypes and genetic markers (single nucleotide polymorphism, dinucleotide repeats) thereby defining the chromosomal region harbouring the genes predisposing to the disease. Association studies are used to analyse in case control series or families a particular marker allele transmitted more frequently than expected under segregation ratio from parents to affected offsprings. The advent of large-scale genomic studies, where several thousand polymorphisms could be measured at once, allow indeed an "uninformed" look at phenotype/genotype correlation. For instance, Gu *et al* reported that *IFRD1* polymorphism may be involved in the severity of CF lung disease through the regulation of neutrophil effector function [96]. The genome wide association approach therefore represents an unbiased yet fairly comprehensive option that can be attempted even in the absence of convincing evidence regarding the function or location of the casual genes. Indeed this kind of analyses is conceptually a revolution whereby several thousand genomic polymorphisms could be correlated to specific phenotype. Understanding phenotypic variation in relation with that found in genotype offers promise to tailor treatment to the best of each patient's condition, setting the basis for "personalized medicine" strategy.

However this kind of studies will require to collect new large cohorts of clinically well-characterized patients, like in CF disease [97]. In addition controlled and standardized investigations of the genetics of susceptibility to infections are very cumbersome in humans because of environmental and other confounding factors (i.e pathogen interaction, incomplete penetrance, population heterogeneity and phenotypic variants) that are numerous and difficult to control in human studies. To address them, patients cohorts will have to be stratified into increasingly smaller groups reducing the efficacy of association analyses. For all these reasons, laboratory mouse models provide a valuable complementary tool in order to dissect the genetic architecture of host susceptibility toward many infectious agents.

B 4.1 Inbred mouse strains model

Experimental animal model are widely and successfully used in order to understand the genetic basis of differential susceptibility to infection.

The advantages in the experimental use of these mouse strains are the accurate reproducibility of several aspects of the human disease, including pathogenesis (tissues and cells involved, types and progression of lesions developed) and physiological responses (inflammation, immunity), and the possibilities to study these functional responses in an environment in which pathogen associated variables such as strain, virulence, dose and route of infection can be carefully controlled [78]. There exist more than 200 commercially available, phylogenetically diverse inbred mouse strains (**Fig.8**) that contain enough genetic diversity to identify individual strains showing major differences in response to a specific infection [98].

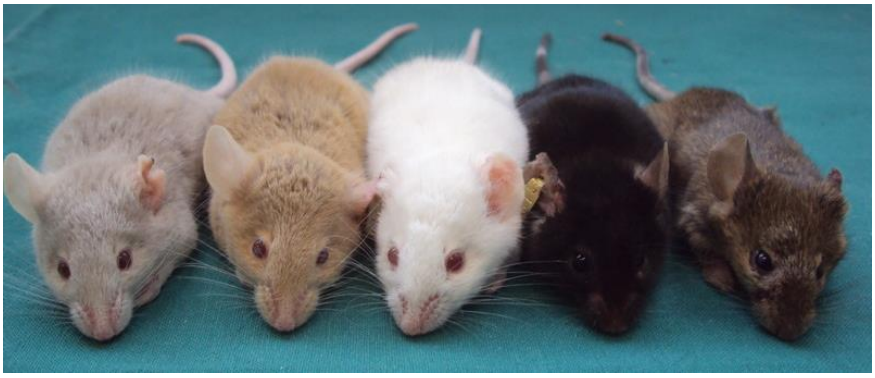

Figure 8: Inbred mouse strains

These inbred strains are genetically pure (homozygous at all their loci with an average at least of 98.6 % after 20 generations) and can therefore be sampled repeatedly allowing multiple serial analyses of different aspects of pathogenesis and host response. The availability of a complete and annotated mouse genome, high-density single nucleotide polymorphism (SNP) maps and the possibility of germ-line modification make the mouse an experimental model of choice for the genetic analysis of susceptibility and response to infection with several types of pathogen.

Genetic studies in mouse models may identify genes, proteins, and biochemical pathways central to the host defence that can be validated in parallel studies in human populations from area of endemic disease.

The principal approaches to identify individual host genes impacting pathogenicity and host response to infections in mice are "reverse genetics" and "forward genetics". The former approach is based on the use of knock-out mice deleted in specific genes in order to dissect their role in host response. Reverse genetics is a classical and largely used analyses method, but carries some inherent limitations which include high embryonic lethality, absence of a detectable phenotype due to genetic redundancy in the pathway targeted or compensatory mechanism [98]. The forward genetic approach is based on the identification of mouse strains that exhibit an unusual or extreme response to infection, being highly resistant or highly susceptible. The genetic component of the trait is then analysed in standard informative crosses to determine inheritance and complexity. In general, a genome-wide scan is conducted to locate the gene in the case of monogenic trait or major quantitative trait loci (QTL) in the case of complex trait.

Actually forward genetic analyses of inbred mouse strains provided several insights into host response to variety of microbial pathogens, including bacteria, viruses and parasites [78], highlighting large difference in susceptibility to infection due to the combinatorial effect of genetic loci and environmental factors. Indeed host genetic variations play a significant role in conferring predisposition to infection. For instance one study described a divergent phenotype among different inbred mouse strains in term of susceptibility to *S. aureus* infection. Whereas C57BL/6 mice were the most resistant in terms of control of bacterial growth and survival, A/J, DBA/2 and BALB/c mice were highly susceptible and succumbed to *S. aureus* infection shortly after bacterial inoculation. Other strains (C3H/HeN, CBA and C57BL/10) exhibited an intermediate susceptibility [99]. A remarkable difference in susceptibility to *Streptococcus pyogenes* infection was found among different inbred mouse strains after intravenous infection with this pathogen. While CBA/J, C3H/HeN, A/J, PwD/Ph mice were the most susceptible strains, C57BL/6J mice displayed an intermediate susceptibility and DBA/2J and BALB/cJ mice were the most resistant strains [100].

Also in the genetic studies of susceptibility to *Candida albicans* infection, mice with different genetic background show a different response. In particular C57BL/6J, BALB/cJ, CBA/J and DBA/1 mouse strains are described as resistant while A/J, DBA/2J NZB/J and AKR/J are described as susceptible to *C. albicans* infection. Focusing on the extreme susceptibility of A/J mice, Tuite and colleagues demonstrated that this phenotype is due to the absence of an adequate inflammatory response describing a partial correlation of susceptibility with a deficiency of C5 complement component [101]. C5 is a downstream component of the activation cascade of complement system. Cleavage of C5 by C5 convertase results in the generation of the potent anaphylotoxin C5a (strong chemoattractant of neutrophils and macrophages) and the formation of C5b-9, the membrane attack complex (MAC) that can directly kill pathogen. In addition, C5 deficiency is associated with susceptibility to other type of infections, including *L. monocytogenes* [102], *Bacillus Anthracis, Aspergillus fumigatus*, but not for example *S. aureus*, in this case the susceptibility of A/J mice is not strictly correlated to C5 deficiency [103].

It was also well described that different mouse genetic background influences the response to influenza A virus infections, highlighting how the course and the outcome of an influenza virus A infection is influenced by several viral and host factors [104] [105].

Finally studies relative to susceptibility to *P. aeruginosa* lung infection described a different susceptibility among different inbred mouse strains, in terms of survival ([106], magnitude of inflammatory response [107], [108], [109], bacterial clearance and lung damage [110].

It is clear that host genetic make-up influence the outcome of infectious diseases. Furthermore the severity of pulmonary disease induced by *P. aeruginosa* may vary from one CF patient to another even carryng the same CFTR mutations, suggest that host genetic background may influence the response to *P. aeruginosa* infection. This also suggests that the host genetic background in a different category of patients at high risk to develop *P. aeruginosa* infection (i.e, immunocompromised, transplants recipients and neutropenic), maybe contribute and provides new insights in dissecting the mechanism at the base of the response to this pathogen.

C. Aim of the Project

Pseudomonas aeruginosa is the fourth most commonly isolated hospital pathogen, multidrug resistant, difficult to-treat and potentially deadly causes of pneumonia. Patients at risk of acquiring *P. aeruginosa* are particularly those with a compromised immune system, due to immunosuppressive therapies or underlying diseases such as cancer, AIDS or the hereditary disease cystic fibrosis (CF). *P. aeruginosa*-host interaction is driven by several factors including the bacterial phenotype and the host genetic make- up. This interaction may be extremely complex and difficult to dissect experimentally. This complexity is based on an equilibrium between the pathogen and the host that mainly depends on: the pathogen factors including life cycles, invasion processes, and specific repertoire of virulence factors, and the host factors including genetic background and defence mechanisms. Therefore both *P. aeruginosa* virulence factors and host genetic background contribute to determining the outcome of the infection that might be clearance of the microbe, asymptomatic colonization and persistence or active infection with the development of resulting pathology.

From the bacterial side, there is still one third of the *P. aeruginosa* genome with no attributed homology to any previously reported sequences with only 6.7% of its genes having a function verified experimentally. Consequently, there is still a lot of information to be uncovered with regards to the mechanisms used by this organism to cause disease. From the host side, there is a remarkable heterogeneity among CF patients in the time of onset as well as in the severity of *P. aeruginosa* lung disease, indicating that the host genetic make-up has major influences in controlling the infection. The genes responsible for such variation are unknown.

The main objectives of this thesis are:

1) to identify and validate novel *P. aeruginosa* virulence factors contributing to disease pathogenesis in a multi-hosts model system. A library of 57,360 *P. aeruginosa* Tn5-mutants was generated and screened for phenotypic virulence traits (swarming, pyocyanin, and protease). A selection of virulence attenuated mutants were then tested for their toxicity in non-mammalian models, namely *Caenorhabditis elegans* and *Drosophila melanogaster.* In this thesis, a selection of Tn5-mutants were tested for invasion and IL-8 production in immortalized respiratory A549 cells. Mortality, inflammation and lung pathology were scored after acute infection of eight selected *P. aeruginosa* mutants in C57BL/6 mice. Five out of eight mutants were significantly attenuated in inducing mortality. This screening generated a list of virulence-related genes relevant for pathogenicity in multi-hosts and favour much-needed insights into aspects of *P. aeruginosa*/host interaction.

2) to seek to which extent the host genetic background may influence the onset, progression, pathophysiology and ultimate outcome of *P. aeruginosa* infection in different inbred mouse stains. In this thesis nine inbred mouse strains, namely A/J, BALB/cJ, BALB/cAnNCrl, BALB/cByJ, C3H/HeOuJ, C57BL/6J, C57BL/6NCrl, DBA/2J, and 129S2/SvPasCR, were infected *P. aeruginosa* by acute infection and scored for body weight, mortality and mean survival time. Based on these results the nine inbred strains were classified for their different response to infection and two deviant clinical phenotype. Next one of the most susceptible and one of the most resistant strains were examined in a time course analysis for their deviant clinical and immunological phenotype in terms of bacterial load, inflammation and lung pathology. Our results indicated that host genetic make-up may have a role in the modulation of cell-mediated immunity playing a critical role in the control of *P. aeruginosa* infection**.**

D. Main Results

D.1 Overview of a strategy for identification and validation of new *P. aeruginosa* virulence factors

In order to identify and validate novel *P. aeruginosa* virulence factors a *de novo* analysis of the whole genome combined with a multifaceted approach, based on a sequential cascade of models (**Fig.9**), was used.

First in collaboration with Prof. Miguel Camara of University of Nottingham, a transposon library was generated using a Tn5 mutagenesis approach that is considered a powerful tool to examine a large number of mutants. Using this technology, it was estimated that around 60.000 mutants could be generated with 95% chance of disrupting any given gene of 327 bps according to Liberati [111]

To perform this mutagenesis pLM1 plasmid was used due to the high transposition efficiency of this Tn5- based vector in *P. aeruginosa* PAO1-L strain. A total of 57,360 Tn5 mutant strains were obtained by conjugation. All the mutants generated were tested individually for attenuations in swarming motility, the loss of the blue pigment pyocianin and reduction in protease production (**Fig.9**). Among a total of 57,360 mutant strains, 404 mutants were found to be attenuated in these virulence factors. These mutants showing pleiotropic phenotypes were tested in a second stage screening by our collaborator of University of Zurich Prof. Leo Eberl using the non-mammalian host models *Caenorhabditis elegans* and *Drosophila melanogaster*. From a total of 404 transposon mutants tested, 114 mutants were mapped in order to identify the insertion sites and to eliminate mutants redundancy. The numbers of mutants with attenuated virulence in at least one non-mammalian model were reduced to 71 candidate genes and to 43 mutants attenuated in both non mammalian host models.

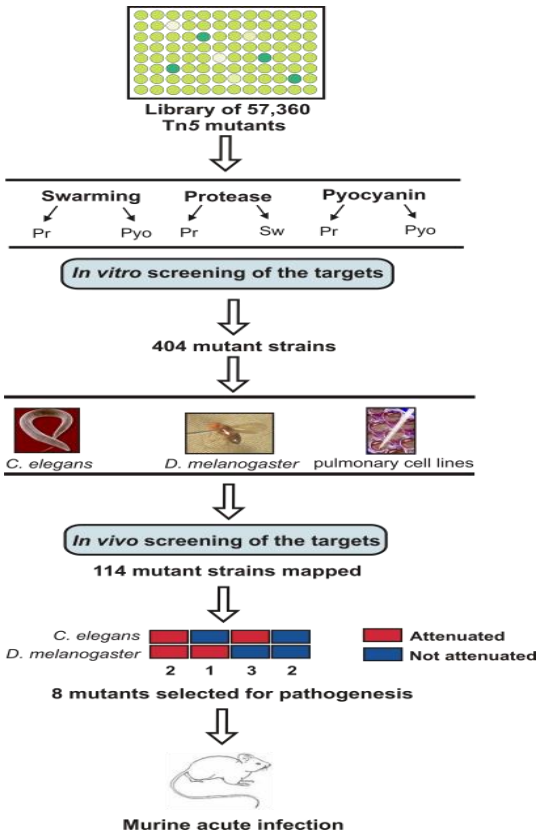

Fig.9 Screening strategy leading to new virulence factors identification and validation

The 43 mutants were analysed for their cytotoxicity by our collaborator Prof. Gerd Doring of University of Tubingen. My work focus on the validation of a selection of 28 attenuated mutants in *in vitro* assays in order to evaluate their invasion capacity and pro- inflammatory induction of IL-8 cytokine in immortalized respiratory A549 cells. The screening generated a list of genes attenuated in the multi-host system listed in a Genomic Target Database and grouped by a ranking score based on the results obtained by phenotypic screening for virulence, validation in *C. elegans*, *D. melanogaster* and *in vitro* models. In addition through an *in silico* analysis, a score was calculated considering the conservation in all sequenced *P. aeruginosa* genomes (LESB58, PA14, PA7, 2192, C3719, PACS2), in *Burkholderia cenocepacia* J2315 and *Escherichia coli* K12, and assigning a negative value in case of homology to human genome.

Finally eight stable in frame deletion mutants were generated by double homologous recombination system for the genes selected, including those not attenuated in virulence as controls, and validated for their virulence in a mouse acute infection model. These genes belong to several functional classes including hypothethical, catabolism regulation (catabolite repression control protein, *crc*), iron uptake (heme oxygenase, *bphO*), quorum sensing (3-oxo-C12-homoserine lactone

acylase, *pvdQ*). In addition, the previous screening identified also five mutants highly attenuated in all the assays and conserved among all *P. aeruginosa* sequenced genomes. Being unknown or poorly studied, these genes could be relevant to further dissect *P. aeruginosa* virulence, and might represent novel targets for future antimicrobials development. The results of these five mutants are not shown in this thesis because of patent issues.

D1.1 Validation of 28 selected *P. aeruginosa* mutants in immortalized cell lines

We screened 28 *P. aeruginosa* Tn5-PAO1 mutant strains, selected by the cytotoxicity assay, for their capacity to invade the immortalized cell line A549 and to induce IL-8 release. An antibiotic exclusion assay was used to evaluate bacteria internalized in the cells after infection. As to IL-8 release, it was measured in the supernatant of cells infected with the mutants by ELISA.

Results show that 19 Tn5-PAO1 mutants were significantly attenuated in their capacity to invade A549 cells in comparison to the wild type PAO1 (**Fig.10A**) (PAO1-L wild type vs PA3867 and PA2414, $p<0.05$; PAO1-L wild type vs PA4916, $p<0.01$; PAO1-L wild type vs PA0914, PA2873, PA5332, PA2385, PA5203, PA4767, PA1634, PA3449, PA1799, PA4768, PA3512, PA3799, PA3761, PA4116, PA4059 and PA5156, $p<0.001$, Student's t-test).

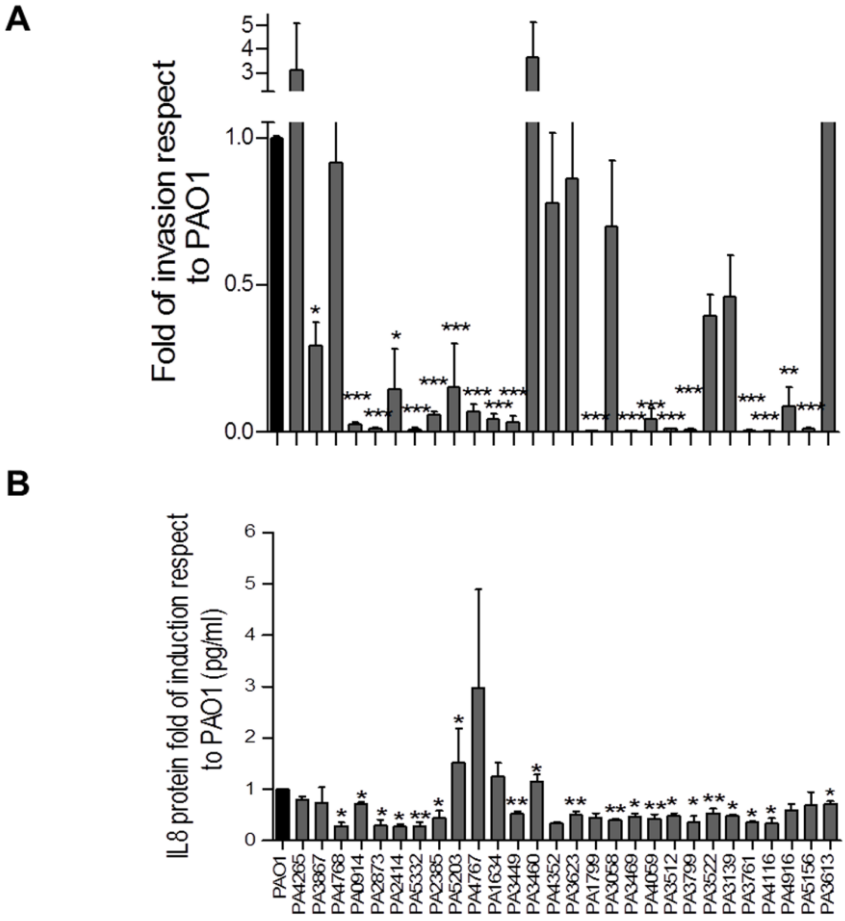

Fig.10 Invasion and IL-8 release in A549 cells. **A)** Invasion and **B)** IL-8 release were evaluated in A549 cells, respectively by antibiotic exclusion assay and by ELISA, after infection with PAO1-L wild type and Tn5 mutants. In details, 1.6 x 10^5 A549 cells/well for the antibiotic exclusion assay and 6 x 10^5 A549 cells/well for the supernatant collection assay were infected with PAO1-L wild type and the Tn5 mutants in exponential growth at multiplicity of infection (MOI) of 100 for antibiotic exclusion assay, and at MOI of 1 for IL-8 measure. After two hours, we treated the samples with polymyxin B for the antibiotic exclusion assay at final concentration of 100µg/ml and amikacin for IL-8 measure at final concentration of 1mg/ml. The abovementioned antibiotic concentrations have demonstrated killing activity exclusively against extracellular bacteria, thus preserving internalized bacteria. For the invasion assay after 2 hours of antibiotic and after osmotic lysis of the cells, we plated serial dilutions of the samples on TSA. The day after, colony forming unit (CFU) were counted to determine the number of bacteria internalized in the cells. For IL-8 measure, after 24 hours we collected and centrifuged the culture media and stored the supernatants at – 80°C. Samples were then assayed by ELISA. Data, from three independent experiments performed in triplicates, are expressed as mean +/- SD. *p<0.05, **p<0.01, ***p<0.001 in the Student's t-test.

IL-8 release was significantly attenuated in A549 cells infected with 20 Tn5-PAO1 mutants in comparison to those infected with wild type PAO1 (**Fig.10B**) (PAO1 wild type vs PA3622, PA0914, PA2873,PA2414, PA2385, PA5203, PA3460, PA4768, PA3512, PA3799, PA3139, PA3761, PA4116 and PA3613 $p<0.05$; PAO1 wild type vs PA5332, PA3449,PA3623, PA3058, PA4059, PA3522 $p<0.01$, Student's t-test). Overall, 17 Tn5-PAO1-L mutants were attenuated both in invasion and IL-8 release in comparison to the wild type PAO1-L.

For the next stage of analysis eight in frame stable deletion mutants (ko) were constructed by double homologous recombination in order to confirm their role in virulence. Mutants were checked

by sequence analyses and were further validated using quantitative *in vitro* analysis for attenuation in virulence traits, *C. elegans* and *D. melanogaster* killing curves, cell invasion and cytokine production assays. The selected mutants were *pvdQ* and *crc* as they were attenuated in *in vitro* assays and in non-mammalian models, *kdpB, bphO* and PA2414 as they were attenuated in all *in vitro* screenings and in non-mammalian models except for *D. melanogaster* model, PA4916 as it was attenuated in all *in vitro* and *in* non- mammalian models except for *C. elegans* model and finally PA5156 and PA3613 as they did not show attenuation in both non-mammalian host models but showed again different levels of attenuation in the others *in vitro* assays. In particular, the results of invasion and secretion of the pro-inflammatory cytokine IL-8 in A549 cells challenged with the eight mutants are shown (**Fig. 11**). Seven out of the selected ko mutants were significantly attenuated in term of invasion capacity (PAO1-L wild type vs *kdpB, pvdQ, bphO, crc* and PA5156 $p<0.001$; PAO1-L wild type vs PA4916 $p<0.01$; PAO1-L wild type vs PA2414 $p<0.05$) and six mutants were attenuated in IL-8 release (PAO1-L wild type vs *bphO* and PA5156 $p<0.01$; PAO1-L wild type vs *kdpB, pvdQ, crc* and PA2414 $p<0.05$) in comparison to the wild type PAO1-L (**Fig.10A** and **B**). PA3613 resulted not attenuated in invasion capacity and IL-8 protein release. Finally these mutants were tested in the mouse model.

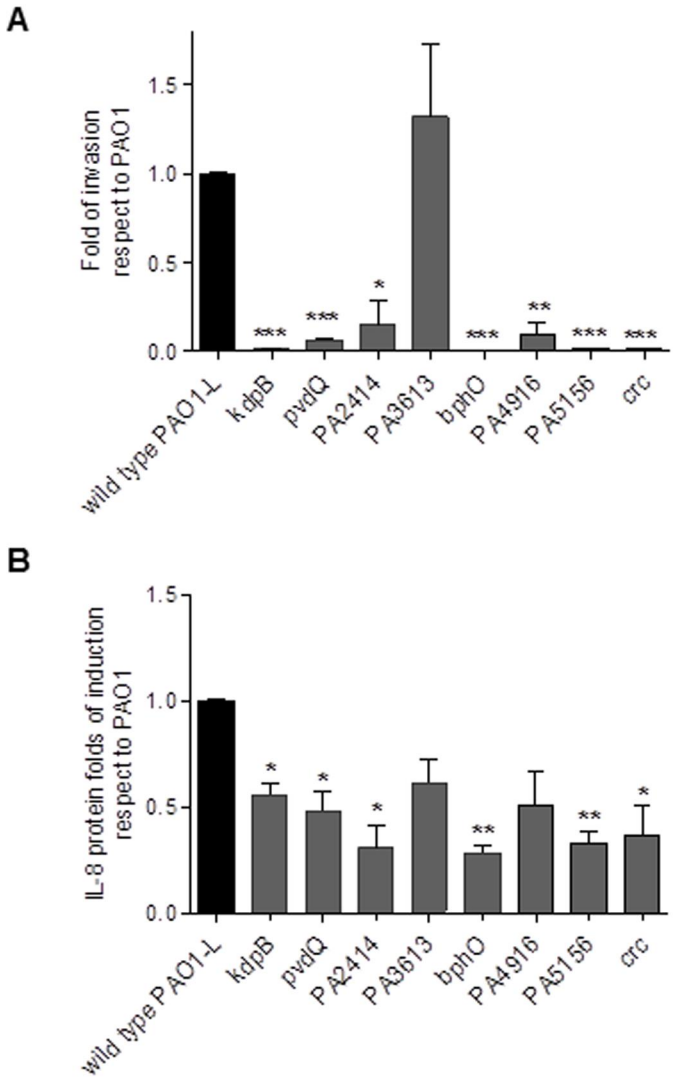

Fig.11 Invasion and IL-8 release in A549 cells of 8 selected Tn-5 mutants. **A)** Invasion evaluated by antibiotic exclusion assay and **B)** IL-8 release by ELISA were evaluated in A549 cells after infection with wild type and ko mutants. Data, from three independent experiments performed in triplicates, are expressed as mean +/- SD. *p<0.05, **p<0.01, ***p<0.001 in the Student's t-test.

D1.2 Validation of eight *P. aeruginosa* mutants in a mouse model of acute lung infection

A mouse model of acute lung infection, was established in C57BL6/NCrlBR mice by intra-tracheal injection of planktonic bacteria to screen the eight stable deletion mutants selected from the previous screenings *in vitro* and in non- mammalian models. First a dose response assay was performed using wild type PAO1-L to choose the correct dose for the screening. So, escalating doses ranging from $5x10^5$ to 10^7 CFU of wild type PAO1-L were used to challenge male C57BL6/NCrlBR mice to determine the relative range of susceptibility (**Fig.12**). The dose of $5x10^6$ CFU/mouse caused mortality in 80% of the infected mice and it was chosen as the first lethal dose.

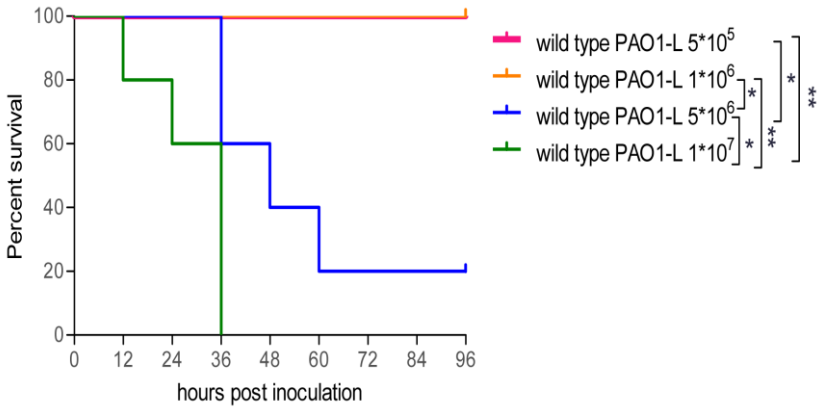

Fig.12 Lethality of different doses of wild type PAO1-L in a mouse model of acute lung infection in C57BL6/NCrl mice. C57BL6/NCrl eight weeks old purchased from Charles river laboratory, were anesthetized with 375 mg/kg of Avertin (2,2,2-tribromoethanil), intubated with a 22-gauge venous catheter and intratracheally injected with different doses of planktonic wild type PAO1-L and survival monitored up to 96 hours (* p <0.05 **p<0.01, Mantel-Cox test).

Next, C57BL/6NCrlBR mice were intratracheally injected with the dose of 5 x 10^6 of type PAO1-L and mutants and survival monitored up to 96h (**Fig.13A**). While wild type PAO1-L was totally lethal within 36h, the lethality of mutants was significantly lower and temporally shifted (**Fig.13A**). Five ot of eight mutants were significantly attenuated in inducing mortality when compared to wild type PAO1-L (wild type PAO1-L vs *pvdQ*, *bphO* and *crc* p<0.001; wild type PAO1-L vs PA4916 and PA5156 p<0.05; Mantel-Cox test) (**Fig.13A**).

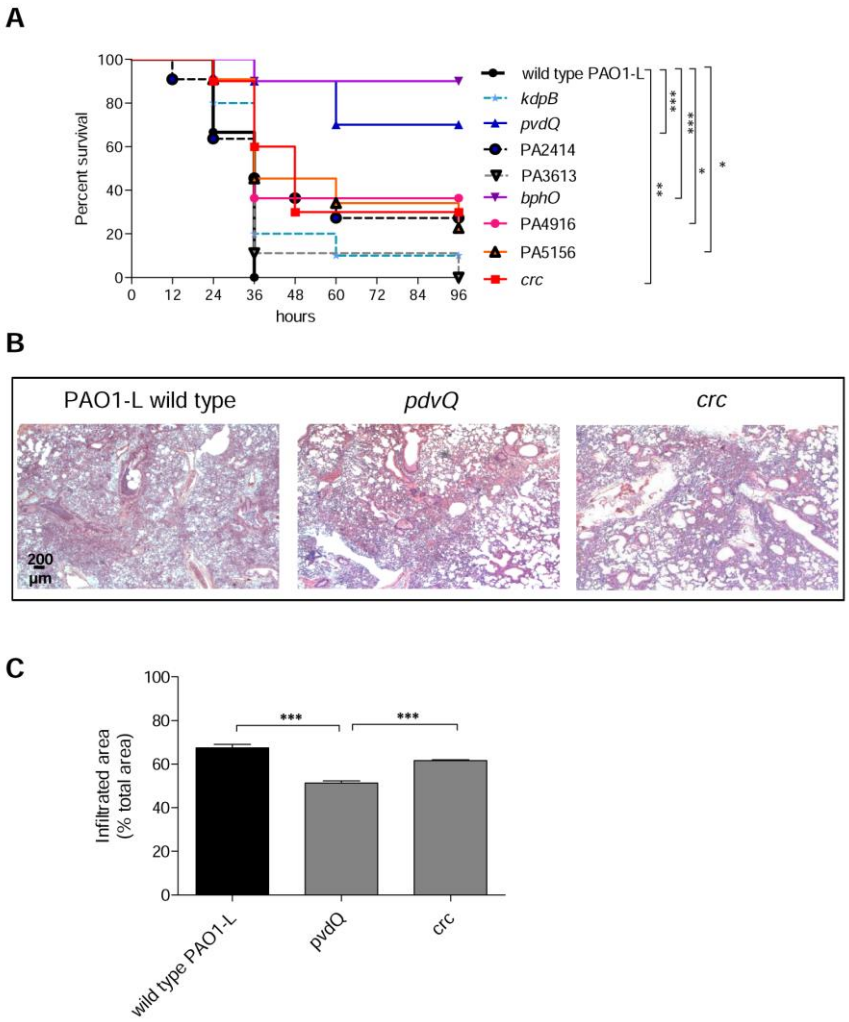

Fig.13 Virulence of *P. aeruginosa* PAO1-L and a selection of mutants in a mouse model of acute lung infection in C57BL/6NCrl mice. C57BL6/NCrl eight weeks old purchased from Charles river laboratory were intratracheal injected with $5x10^6$ CFU of PAO1-L wild type or mutants. **A**) Survival was monitored up to 96 hours. Two independent experiments were pooled. Statistical analysis was calculated for pair wise comparisons between wild type and mutant strains. *p<0.05, **p<0.01, ***p<0.001, Mantel-Cox test. **B**) Lung histopathology was performed after 24h from infection for wild type PAO1-L, *pvdQ* mutant and *crc* ko mutant. **C**) Quantification of infiltrated areas as a percentage of total tissue area with mean ± SEM is shown. Statistical analysis was calculated for pair wise comparisons between wild type and mutant strains. ***p<0.001, Mann-Whitney.

Furthermore, histopathological analysis **(Fig. 13B**) showed that wild type PAO1-L strain induced slightly highest inflammation and haemorrhage in comparison to *crc* and *pdvQ* mutants. The area infiltrated by inflammatory cells was significantly higher in the lungs infected by wild type PAO1-L in comparison to the *pvdQ* mutant (wild type PAO1-L and *crc* vs *pdvQ* p<0.001, Mann Whitney test) (**Fig.13C**).

D.2 Genomic Target Database of *P. aeruginosa* virulence factors

Overall all the results obtained from this sequential screening model were used to generate a genomic target database. An exemplificative picture of this database is shown below (**Table 1**). All listed factors in the database are genes involved in virulence identified by insertional mutagenesis and may be essential for *in vivo* infection. In addition sequence analyses showed that these genes are conserved among strains of different origin, thus representing good alternative candidates targets for antibiotic therapies in the future.

So far, 28 Tn5 and eight stable deletion mutants were validated in different model systems and five stable deletion mutants were attenuated in murine model. It should be noted that some mutants attenuated in *C. elegans* were not attenuated in the mouse model. Our data thus indicate that identification of virulence genes carried out mainly *in vitro* does not imply that they are relevant for their pathogenesis *in vivo*, suggesting a host-specific response to *P. aeruginosa*, and the necessity to test a selection of mutants in the mouse model, as the nearest to the human host.

	Gene name	Class of confidence	In silico analysis			*In vitro* phenotypic analysis			Non mammalian models		Screening in immortalized cells				Murine model	Final Score
			Homology (range) to all sequenced *P. aeruginosa* genomes*	Homology to *B. cenocepacia* J2315	Homology to human genome	Protease production	Swarming motility	Pyocyanin production	*C. elegans*	*D. melanogaster*	Bacteria cytotoxicity in A549	SN cytotoxicity in A549	Invasion In A549	IL-8 production in A549	Acute murine lung infection	Conservation/ phenotypic/ validation
PA2385	pdvQ	1	94-99%	NSH	NSH	+	+	+	+	+	+	-	+	+	+	10
PA2414	L-sorbosone dehydrogenase	2	94-99%	25%	NSH	+	+	+	+	-	+	+	+	+	-	10
PA1634	kdpB	2	98-100%	60%	28%	+	+	+	+	-	+	+	+	+	-	10
PA5332	crc	1	100%	40%	29%	+	+	-	+	+	+	+	+	+	+	9
PA4116	bphO	1	85-100%	NSH	NSH	+	-	+	+	-	+	+	+	+	+	9
PA4916		4	97-99%	66%	NSH	+	+	+	-	+	+	-	+	+	+	9
PA5156		4	94-99%	26%	NSH	+	+	+	-	-	nd	nd	+	+	+	7
PA3613		4	97-100%	NSH	NSH	-	+	+	-	-	-	-	-	-	-	4

**P. aeruginosa* sequenced genomes (LESB58, PA14, PA7, 2192, C3719, PACS2)
NSH not significant homology
nd not determined
+ attenuated in comparison to wild type PAO1
- not attenuated in comparison to wild type PAO1

Table 1. Genomic target database (GTD) generation. The genomic target database was generated by attributing a score to the results obtained by phenotypic screening for virulence, validation in *C. elegans*, *D. melanogaster*, *in vitro* models and murine model of acute lung infection, and to the *in silico* analysis.Score criteria: first, a score for homology to each sequenced genome was evaluated, then an overall score was assigned to each mutant as indicated by following criteria. Homology to single *P. aeruginosa* sequenced genome (LESB58, PA14, PA7, 2192, C3719, PACS2), score 1(90-100%), score 0,5(75-90%), score 0 (0-75%). Overall homology score, score 2 (sum of all conservation scores ≥5,5) score 1,5 (sum of all conservation scores = 4,5-5), score 1 (sum of all conservation scores 4-3,5) score 0,5 (sum of all conservation scores ≤ 3&>0) score 0 (sum of all conservation scores= 0) Homology to B. cenocepacea J2315 genome: score 1(70-100%) score 0,5 (55-70%), score 0 (0-55%). Homology to E. coli k 12 genome score 1(70-100%) score 0,5 (55-70%), score 0 (0-55%). Homology to human genome, score 0 (no homologs) score -1(presence of homologs)

D.3 Survival and body weight of *P. aeruginosa*-infected inbred mice is strongly dependent upon genetic background.

For the second aim of my thesis, nine different inbred mouse strains, namely A/J, BALB/cJ, BALB/cAnNCrl, BALB/cByJ, C3H/HeOuJ, C57BL/6J, C57BL/6NCrl, DBA/2J, and 129S2/SvPasCRL were infected with $5x10^6$ CFU of planktonic *P. aeruginosa* clinical isolate AA2 via intra-tracheal injection, and monitored for change in body weight and mortality over a period of seven days. As shown in **Fig.14,** a wide-range of survival and weight loss were observed among different inbred mice. The most deviant survival phenotypes were observed for A/J, 129S2/SvPasCRL and DBA/2J showing high susceptibility and BALB/cAnNCrl and C3H/HeOuJ showing more resistance to *P. aeruginosa* infection. BALB/cJ, BALB/cByJ, C57BL/6J, C57BL/6NCrl showed intermediate phenotype. In more detail, susceptible A/J, 129S2/SvPasCRL and DBA/2J died within the first two days of infection, showed a mean survival time of around one day and a rapid and fatal decrease of body weight (**Fig. 14A, B** and **C**). Within the susceptible mice, A/J were significantly different compared to DBA/2J and 129S2/SvPasCRL showing a faster decrease of body weight at day one (Table S1, submitted paper) and a kinetic of death significantly more rapid (A/J vs DBA/2J, $p < 0.01$; vs 129 S2SvPAsCrl , $p<0,05$ Mantel-Cox test) (**Fig.14A**). Resistant mice BALB/cAnNCrl and C3H/HeOuJ showed a significant lower susceptibility to *P. aeruginosa* infection compared with A/J, DBA/2J and 129S2/SvPasCRL with cases of survival(C3H/HeOuJ and BALB/cAnNCrl vs 129S2SvPasCrl, $p<0.001$; vs DBA/2J $p<0.01$; vs A/J $p< 0.0001$, Mantel-Cox test), a mean survival time of at least three days and a progressive weight recovery of the survivors at day seven (**Fig.14**). Within the resistant mice, BALB/cAnNCrl and C3H/HeOuJ were not significantly different for the body weight, kinetic of death and survival time (**Table S1** of submitted paper and **Fig. 14**). The above described differences in resistance and susceptibility of most deviant inbred mice were confirmed by infecting with a lower *P. aeruginosa* infection dose of $5x10^5$ CFU(**Table S2** and **Figure S1,** submitted paper).

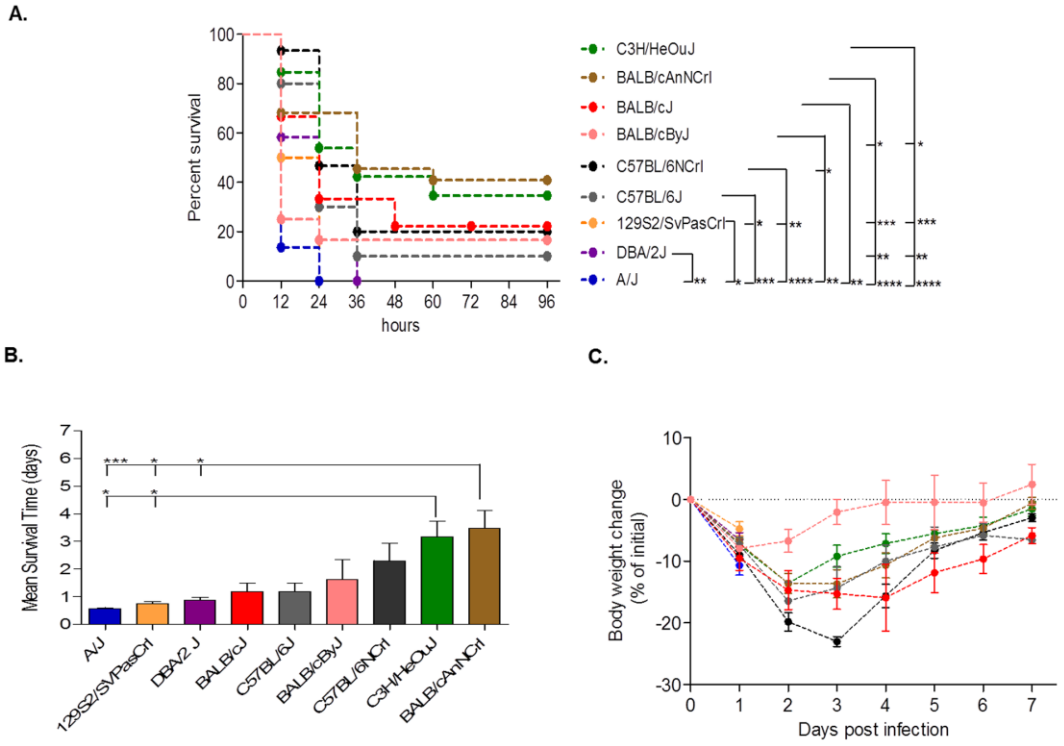

Fig. 14 Survival, body weight and mean survival time after *P. aeruginosa* infection in inbred mouse strains.
A/J (n=22), BALB/cJ (n=9), BALB/cAnNCrl (n=8), BALB/cByJ (n=12), C3H/HeOuJ (n=26), C57BL/6J (n=10), C57BL/6NCrl (n=15), DBA/2J (n=12), and 129S2/SvPasCRL (n=12) mice were inoculated with $5x10^6$ CFU of *P. aeruginosa* clinical isolate AA2, and monitored for survival (**A**) and weight change for a period of seven days after infection (**C**). In addition, mean survival time was calculated based on the survival curve (**B**). The data are pooled from two to four independent experiments. Statistical significance by Mantel-Cox test for survival (**A**), One.way ANOVA with Bonferroni's Multiple comparison test (**B**) for mean survival time (*p<0.05, **p<0.01, ***p<0.001). Statistical significance by Two-way ANOVA, with Bonferroni' Multiple comparison test for body weight is reported in **Table S1A**.

D4. Impaired cell-mediated immunity leads to faster replication of *P. aeruginosa* in A/J mice when compared to C3H/HeOuJ.

Next, one of the most susceptible and resistant mouse strains were characterized for their deviant clinical and immunological phenotypes after *P. aeruginosa* AA2–induced acute pneumonia. The *P. aeruginosa* load and immune response of infected mice in terms of leukocyte recruitment, myeloperoxidase activity, and local cytokine production in the airways were investigated in A/J and C3H/HeOuJ mice during an early time course (6, 12 and 18 hours post-infection). Starting from a challenge of $5x10^6$ CFU, significant increase of total bacterial load of 2 $\log_{10}$ CFU ($4.1x10^8$) at 18 hours in the lung of susceptible A/J mice was observed indicating an uncontrolled replication of bacterial cells (**Fig. 15A**). Conversely at the same time points, the bacterial load in the lungs of resistant C3H/HeOuJ mice was unchanged in respect to the initial inoculum (6.9×10^6) suggesting that resistant mice are able to keep in check the infection. A/J susceptible and C3H/HeOuJ resistant mice were significantly different in their bacterial load at all-time points(A/J vs C3H/HeOuJ,

p<0.001, Mann-Whitney U test). Similar differences were also observed in different airways compartments as assessed by Bronco Alveolar lavage Fluid (BALF) (**Fig. 15B**) and lung homogenate analysis (**Fig. 15C**).

Leukocyte recruitment in the BALF of susceptible A/J mice early after *P. aeruginosa* infection was significantly lower than C3H/HeOuJ resistant mice (**Fig. 15D**). Furthermore, while the low leukocyte numbers remained stable in A/J during 18 hours, their recruitment in C3H/HeOuJ increased and reached the pick at 12h.

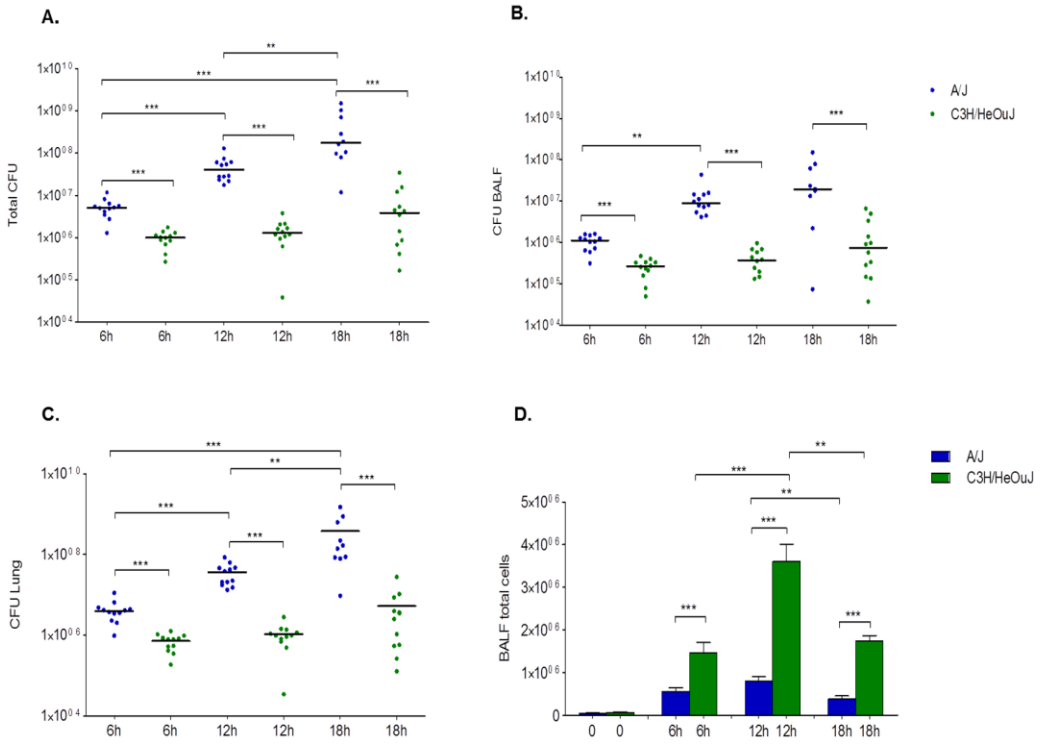

Fig. 15 Early time course of *P. aeruginosa* load and leukocytes recruitment in BALF and lung of susceptible A/J and resistant C3H/HeOuJ mice. A/J (n=12, for each time) and C3H/HeOuJ (n=12, for each time) mice were challenged with $5x10^6$ CFU of AA2 clinical stain and analysed during a time course post-infection. Bacterial loads in the total lung (**A**) including broncoalveolar lavage (BAL) (**B**) and lung homogenate (**C**) were counted at 6, 12 and 18 hours in surviving mice. BAL fluid was extracted with a 22-gauge venous catheter by washing the lungs with RPMI with protease inhibitors. Dots represent CFU per lung in individual mice and horizontal lines represent median values reported in log scale. Total leukocytes were analyzed in BALF of *P. aeruginosa* infected mice (**D**). Bars represent median values. Blue is referred to A/J and green to C3H/HeOuJ. The data are pooled from two independent experiments. Statistical significance by Mann-Whitney U test is indicated: **p<0.01, ***p<0.001, ****p<0.0001.

In particular, a significant increase in neutrophil levels for C3H/HeOuJ, compared to A/J was observed during 18h (p<0.001, Mann-Whitney U test) (**Fig. 16A**). The higher level of MPO activity in the BALF of C3H/HeOuJ compared to A/J supported these data (**Fig. S2,** submitted paper).

Macrophages were also significantly higher in the lung of C3H/HeOuJ mice compared to A/J mice at 12 hours post infection (p<0.05, Mann-Whitney U test), but no striking differences were present

at 6 and 18 hours post infection (**Fig. 16B**). Lymphocytes and epithelial cells were not statistically different during the time course analysis (**Fig. 16C** and **D**).

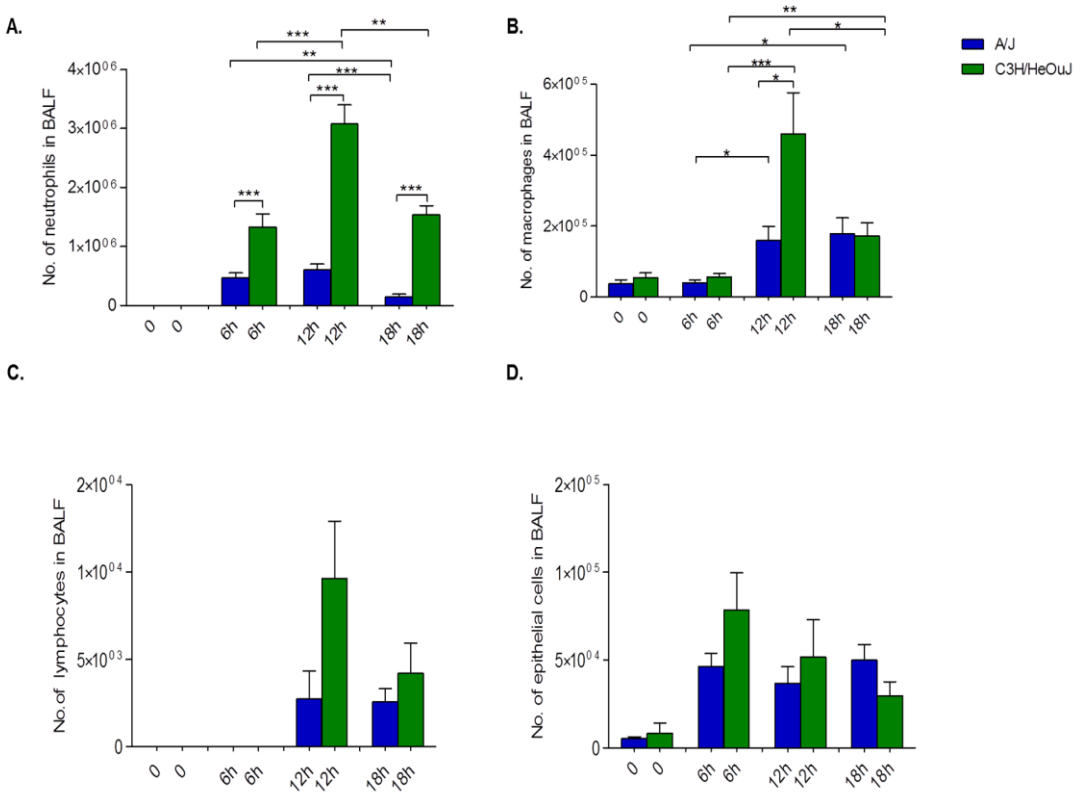

Fig.16 : Lung inflammatory response after *P. aeruginosa* infection in susceptible A/J and resistant C3H/HeOuJ.
The number of neutrophils (**A**), macrophages (**B**), lymphocytes (**C**) and epithelial cells (**D**) recruited in the airways were determined in the BALF from A/J (n=12) (blue bar) and C3H/HeOuJ (n=12) (green bar) mice after 6, 12 and 18 hours of *P. aeruginosa* infection with $5x10^6$ CFU of AA2 clinical isolate. Differential cell count was performed on cytospins stained with Diff Quick The data are pooled from two independent experiments. Statistical significance by Mann-Whitney U test is indicated: *p<0.05, **p<0.01, ***p<0.001.

When CFUs and neutrophils recovered in the BAL fluid were plotted together, a distinct trend was observed in A/J and C3H/HeOuJ mice during 18 hours of infection. **Fig 17** showed a lower number of neutrophils recruited and a higher number of *P. aeruginosa* CFUs in A/J mice in comparison to C3H/HeOuJ mice. In particular, looking at the ratio of *P. aeruginosa* CFUs and neutrophils, significant differences were found (**Fig S3,** submitted paper**)**. During the whole time-course, the ratio between CFUs and neutrophils was significantly lower for C3H/HeOuJ mice when compared to A/J mice, indicating a higher capacity of C3H/HeOuJ mice to control *P. aeruginosa* infection.

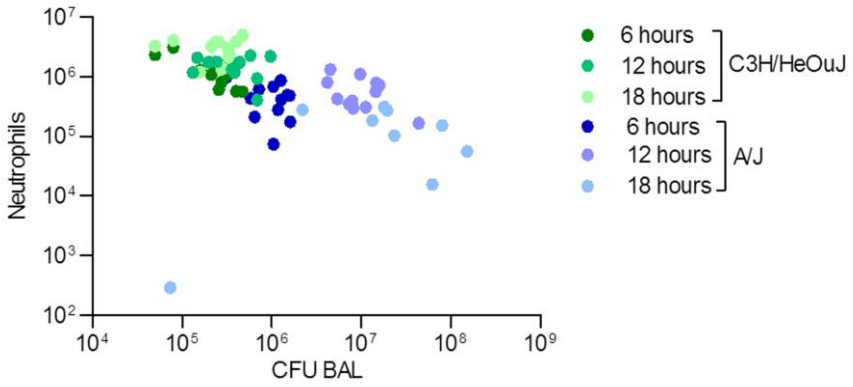

Fig.17 Correlation of CFU with neutrophils in the BAL fluid after *P. aeruginosa* infection in susceptible A/J and resistant C3H/HeOuJ mice. CFUs and neutrophils recovered in the BAL were plotted for the two murine strains during 18 hours of *P. aeruginosa* infection. Blue dots represent A/J mice (n= 12, for 6 and 12 hours, n= 9 for 18 hours) and green dots C3H/HeOuJ mice (n= 12 for each time). The data are pooled from two independent experiment.

D4.1 Cytokines and chemokines profile in the airways of A/J and C3H/HeOuJ mice

To better characterize the airway inflammatory response of the two deviant inbred mouse strains, we measured the concentration of a large panel of twenty-three cytokines and chemokines in murine lung homogenates. As shown in **Table 2**, the overall levels of proinflammatory citokines were significantly higher in the lung of A/J mice respect to C3H/HeOuJ mice and reach significance for interleukin 1α (IL-1α), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin12p40 (IL-12p40), interleukin 13 (IL-13), interleukin 17 (IL-17), granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ), murine Keratinocyte chemoattractant chemokine (mKC), Monocyte Chemoattractant Protein 1 (MCP-1), Machrophage Inflammatory Protein-1 alpha/beta (MIP-1α and MIP-1β) in at least one time point. Other cytokines, tumor necrosis Factor α (TNF-α) and interleukin 5 (IL-5), were higher in A/J compared to C3H/HeOuJ mice but did not reach significance. Thus, A/J mice showed an excessive release of pro-inflammatory cytokines that does not correlate with cellular response when compared to C3H/HeOuJ mice.

	A/J			C3H/HeOuJ			A/J vs C3H/HeOuJ		
Cytokines	**6h**	**12h**	**18h**	**6h**	**12h**	**18h**	**6h**	**12h**	**18h**
IL1α	777.405	443.0235	157.775	378.485	489.875	241.995	*	ns	*
IL1β	726.39	1151.41	393.915	462.985	492.23	302.1	ns	ns	ns
IL2	nd	nd	nd	nd	nd	nd	-	-	-
IL3	3.485	3.465	1.785	1.505	1.835	1.385	ns	*	ns
IL-4	15.595	13.555	12.085	9.675	12.67	10.725	ns	ns	ns
IL5	8.62	7.42	5.165	4.205	4.05	5.675	ns	ns	ns
IL6	473.33	378.19	193.035	305.56	201.27	176.795	ns	*	ns
IL9	35.7	36.43	48.495	35.705	35.7	55.195	ns	ns	ns
IL10	39.25	49.095	32.04	20.44	30.75	23.51	*	*	ns
IL12p40	56.4	21.655	23.855	14.59	19.8	21.21	*	ns	ns
IL-12p70	254.15	182.17	113.995	164.805	228.87	147.245	ns	ns	ns
IL13	71.26	74.4	73.515	40.14	55.46	65.09	*	*	ns
IL17	18.945	20.26	22.41	9.825	12.41	14.525	*	*	ns
eotaxin	nd	nd	nd	nd	nd	nd	-	-	-
G CSF	1778.86	5840.23	6713.8	607.365	2067.78	3266.77	*	*	ns
GM CSF	170.26	156.01	151.21	124.38	129.5	123.595	ns	*	ns
IFNγ	8.43	6.69	1.97	2.545	7.55	2.105	*	ns	ns
KC	68190	69190	69290	5793.96	6747.85	3068.26	*	ns	ns
MCP1	2826.25	1752.27	1109.07	757.01	1503.73	731.82	*	ns	ns
MIP1α	963.815	711.72	577.235	270.05	487.66	365.4	*	ns	ns
MIP1β	62.63	69.04	58.95	42.735	44.59	37.83	*	ns	ns
RANTES	32.67	45.24	27.125	15.22	25.115	34.03	ns	ns	ns
TNFα	18.08	15.105	16.6	9.76	12.38	15.105	ns	ns	ns

Data are expressed as median of pg/500ug lung.
Nd: not detectable; ns: not significant.

Table 2. Cytokines and chemokines levels in lung homogenates of susceptible A/J and resistant C3H/HeOuJ mice infected with *P. aeruginosa* during a time course. Total lung homogenates protein content was quantified with Bradford's assay at the final concentration of 500 mg/ml. A panel of 23 chemokines and cytokines were meausered using Bio-Plex pro ™ Mouse Cytokine Standard 23-Plex, Group I. Statistical analysis for comparison of A/J vs C3H/HeOuJ at each time point by the non-parametric Mann-Whitney U test (*$p<0.05$) is reported.

D4.2 Pathological differences in A/J and C3H/HeOuJ mice during acute pneumonia

The histopathologic analysis of *P. aeruginosa* AA2–induced acute pneumonia revealed striking differences between A/J and C3H/HeOuJ mice (**Fig. 18** and **Fig. S4**). During an early time course a fast and consistent recruitment of inflammatory cells in C3H/HeOuJ mice compared to a delayed and lower recruitment in A/J was observed (**Fig. 18 A-C, E-G**). More in detail, during early phases of infection pathologic scores showed that the high inflammatory response of C3H/HeOuJ resistant mice was characterized by strong polymorphonuclear leukocytes (PMNs) recruitment in BALF and in the interstitial areas of the lungs followed by a progressive increase in macrophages involvement and a moderate expansion of bronchus associated lymphoid tissue (BALT)-like structure (**Fig. 18 A-C, E-G** and **Fig. S4**). These cell-mediated response may have a key role in mounting an effective response to control bacterial clearance. Indeed, immunofluorescence staining showed low number of *P. aeruginosa* cells in the lungs of C3H/HeOuJ mice (**Fig. 18H**). The susceptible A/J strain responded in a delayed manner characterized by a lower cell recruitment associated to low damage in the lung, and absence of a relevant interstitial/BALF response (**Fig. 18 A-C, E-G** and **Fig. S4**). The BALT-like structure observed in C3H/HeOuJ mice was substantially never observed in A/J susceptible strain. Indeed, immunofluorescence staining showed high numbers of *P. aeruginosa* cells localized both within the bronchial lumen and in the alveolar space indicating inadequate response of A/J mice to control bacterial replication in the lung (**Fig. 18D**).

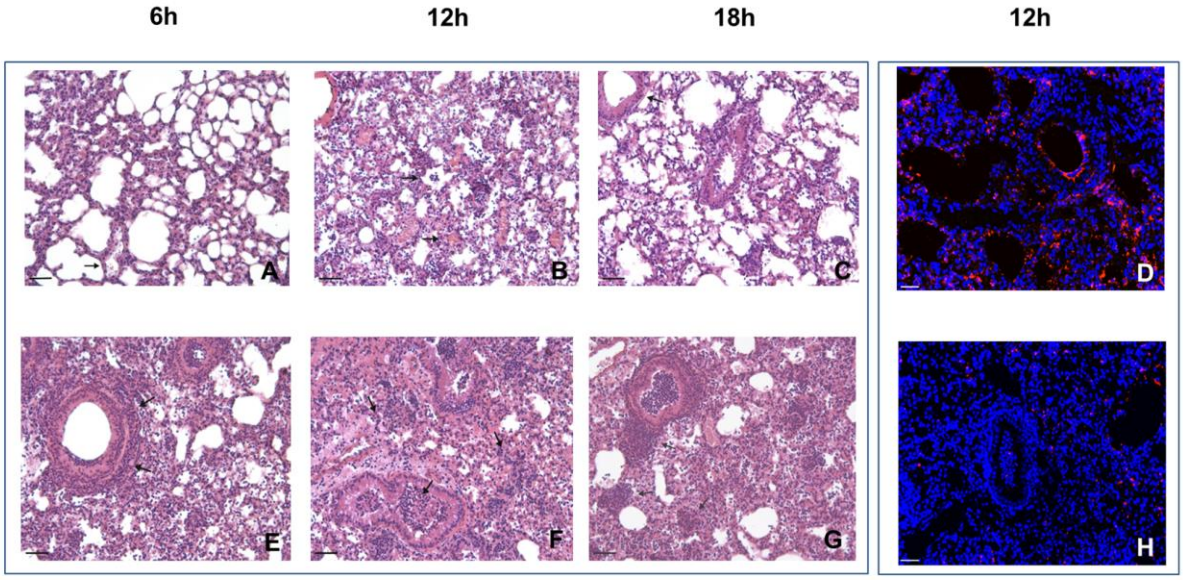

Figure 18 : Histopathology in susceptible A/J and resistant C3H/HeOuJ murine lungs after infection with *P. aeruginosa*. The lungs of A/J (A-D) and C3H/HeOuJ (E-H) were removed, fixed in 10% buffered formalin for at least 24 h and embedded in paraffin. Consecutive 2-µm sections from the middle of the five lung lobes were used for histological and immunofluorescence examination in each mouse. Sections for histological analysis were stained by Haematoxylin-Eosin according to standard procedures (A-C) and in immunofluorescence with specific antibody a rabbit anti serum specific for *P. aeruginosa* and Texas Red labelled goat anti- rabbit IgG as described (red)[51] (D, H). Counterstaining was performed with 49,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (blue). After time course analysis, extensive infection and inflammation were visible in murine lungs with major differences between A/J and C3H/HeOuJ. Bars, 200 µm for H&E images, 100µm for immunofluorescence images. Severity of lesions and lung involvement was scored as reported in Fig S4. Arrows indicate BALT-like structure in C3H/HeOuJ and A/J mice.

E. Conclusions and Future prospects

E1. Identification and validation of novel *Pseudomonas aeruginosa* virulence factors

P. aeruginosa is an opportunistic Gram-negative human bacterium and one of the leading cause of hospital-acquired infections. This pathogen, displaying a multifactorial and combinatorial virulence [33], causes a wide range of acute and chronic diseases in a large category of patients at risk including CF patients. It has been described that *P. aeruginosa* acute infection is characterized by a large arsenal of virulence factors, such as flagella, type III secretion system, LPS and others. In contrast virulence factors required for the initiation of acute infections in CF are selected against during chronic infection leading to less invasive strains [50].

Consistent with its large genome size and environmental adaptability, *P. aeruginosa* contains the highest proportion of regulatory genes observed for a bacterial genome which leads to large and complex phenotypic versatility. Although over the past two decades an enormous progress has been made in the genome analyses approach to identify and characterize virulence factors involved in *P. aeruginosa* infection pathogenesis, only 6.7% of its genes has a function verified experimentally and still one third of its genome has no attributed homology to any previously reported sequence. Consequently, there is still a lot of information to be uncovered with regards to the mechanisms used by this organism to cause disease. Taking into account all these considerations, the first aim of my thesis was to identify novel *P. aeruginosa* virulence factors. We developed a new screening strategy combining an *in vitro* testing for reduced production of virulence factors and validation in a cascade of *in vitro* and *in vivo* models including airways cells, non-mammalian host models (*C. elegans, D. melanogaster*) and mouse model. We identified a total of 404 independent pleiotropic mutants (attenuated in two or more virulence traits). All 404 mutants were screened for reduced virulence in the two non-mammalian infection models and 43 mutants attenuated in both *C. elegans* and *D. melanogaster*, were then tested for their reduced cytotoxicity on A549 alveolar epithelial cells. Screening of these mutants in A549 alveolar epithelial cells identified 28 mutants attenuated in their cytotoxicity potential. Among these mutants 19 were found to be attenuated in invasion capacity and 20 in IL-8 cytokine release. The screening generated at the end a selection of genes differentially attenuated in the multi-host system listed in a Genomic Target Database and grouped by a ranking score based on the phenotypic outcomes and bioinformatics analyses. A comparative protein sequence analysis was carried out against six sequenced genomes (PA14, LESB58, PA7, 2192, C3719 and PACS2) of highly pathogenic *P.*

aeruginosa and against *E. coli* K12 and *B. cenocepacia* J2315 in order to check the rate of conservation and homology to human of these virulence factors. Finally we selected mutants in *pvdQ* and *crc* as they were attenuated in all phenotypes, non-mammalian models and *in vitro* assays, the mutants in *kdpB*, *bphO* and PA2414 as they were attenuated in all models except for *D. melanogaster* model, PA4916 as it was attenuated in all models except for *C. elegans* models and finally PA5156 and PA3613 as they did not show attenuation in the non-mammalian host models but showed again different levels of attenuation in the other *in vitro* assays. Stable in frame deletion mutants were generated by recombination system and validated for their virulence in a mouse acute infection model. Five out of eight mutants (*pvdQ*, *crc*, *bphO*, PA4916 and PA5156) showed attenuated lethality in the mouse model

Through our screening, we were able to isolate a large number of mutants with insertions in genes already demonstrated to be required for *P. aeruginosa* virulence in nematodes and mammals, including genes involved in antibiotic resistance, biofilm formation, virulence factors production, metabolism and quorum sensing regulation. Similar to the *C. elegans* screening of PA14 transposon mutants described by Feinbaum *et al* [112], we identified a number of genes that are involved in adaptation to environmental stress by playing a protective role. Those include a large number of metabolic genes involved in the generation and transport of precursor metabolites and energy (*bphO* and PA2414), genes involved in DNA synthesis and modification (*smpB*, *pdxA*, PA3867, PA3950 and PA4282), genes involved in the catabolism regulation (catabolite repression control protein, *crc*) and quorum sensing (3-oxo-C12-homoserine lactone acylase, *pvdQ*). The identification of a number of mutants corresponding to biofilm formation (*psl*, *mucP* and *pelG*), type IV and VI secretion systems (*xcpT*, *pilD* and *tssA1*), and stress response (*phoR*, *rpoS* and *parR*) also illustrate their importance for survival of *P. aeruginosa* in a host environment with more stringent and harmful conditions. In addition, genes with hypothetical function (PA4916, PA5156 and PA3613) and genes playing a part in amino acid synthesis and metabolism were identified (*pauB1* coding for a D-amino acid oxidase, *hisD* coding for a histidinol dehydrogenase, PA3139 coding for a threonine-phosphate-decarboxylase).

In the screening cascade we observed that two mutants out of the eight (PA5156, PA3613) selected for their pleiotropic phenotypes were not attenuated in the disease models *C. elegans* and *D. melanogaster*. In addition, while we have found accordance between presence or absence of attenuation for some targets in *C. elegans* and in the murine model (*kdpB*, *pvdQ*, *bphO*, *crc*, PA3613), PA2414 was attenuated in *C. elegans* but not in mice and PA4916 and PA5156 were attenuated in the mouse model of acute infection, but not in *C. elegans*. Other incongruities were observed between the results obtained in mice and those from other screenings. A study by Tan and

coworkers [113] identified, by screening of 2,400 TnphoA mutants, genes products corresponding to virulence factors. They demonstrate that these mutants were less pathogenic in nematodes, plants and mice with some differences in term of attenuation between the *C. elegans* and mouse models, as observed for some mutants in our screening models. Our data indicate that identification of virulence genes carried out mainly *in vitro* and in non-mammalian host model does not imply that they are relevant for their pathogenesis *in vivo*. The use of non-mammalian infection models has several critical aspects, as the temperature for the cultivation of the nematode that affects the expression of certain virulence factors, the absence of the target organ and the lack of specific receptors or pathways. However, although mice are the first choice for understanding infectious diseases in human, screening a large amount of mutants in mouse models is unfeasible. For these reasons non-mammalian models remain useful surrogate hosts. In addition to being largely used to identify virulence factors, *C. elegans* has been exploited to study responses to infection also with other pathogen like *Enterococci* [114] and *Staphilococcus aureus* [115], as well as to compare the virulence of *P. aeruginosa* clinical isolates together with *D. melanogaster* and *G. mellonella* models [116]. Furthermore, pathways conserved in vertebrates characterize *Drosophila* response to pathogens, and mammalian and *C. elegans* innate immune defenses [117] [118].

Taken together our results suggest that, using a genomic approach devised to screen the entire *P. aeruginosa* genome for novel virulence genes and a multifaceted approach based on a sequential cascade of models for the validation step, we were able to identified several novel virulence genes. These genes should be further investigated for their function and could represent interesting targets for an innovative anti-virulence approach to *P. aeruginosa* infections. Several studies [119] [120] [121] describe a novel therapeutic approach for infectious disease interfering with the ability of the bacteria to activate specific virulence traits that are needed to establish infection, without killing or inhibiting bacterial growth, or with the host-pathogen interaction. By preventing the expression or activity of virulence traits, the bacteria might be less able to colonize the host and less pathogenic. Targeting bacterial virulence is attractive since it is specific toward pathogenic bacteria. In addition, as this strategy does not directly kill the bacteria, there is presumably less evolutionary pressure for the development of resistance than with traditional antibiotics. This inhibition could also allow the host immune system, including the normal microbiota, to prevent bacterial colonization or clear any established infection. Some concerns should be considered about anti-virulence therapy for *P. aeruginosa* infections. *P. aeruginosa* virulence is a highly complex, multifactorial process requiring the coordinated activity of many bacterial gene products which can be widely different depending on the infection is established. The idea of a core set of virulence factors common to all infection models is unlikely. The spectrum of virulence factors that play a role in a given host model depends

on a wide variety of factors including the characteristics of the site of infection, the type of the immune response and the phase of infection, and even host response. Virulence factors playing a role in distinct types of *P. aeruginosa* infection in humans could be different, implicating a great effort to personalize the treatment and thus posing a challenge for the development of new therapeutics.

Another concern deals with the anti-virulence approach in CF chronic infection. It has been shown that long-term colonization of the CF host is maintained by *P. aeruginosa* patho-adaptive lineages, which are clonal with the initially acquired strain and carry phenotypic variants [50]. A number of genetic mechanisms are responsible for generating clonal variants in *P. aeruginosa*. Most CF strains consistently acquire common mutations in virulence genes including motility genes, quorum-sensing regulator, the type-III secretion system, the multidrug-efflux pump and in mutator phenotypes [51] [75]. Interestingly, virulence factors required for the initiation of acute infections are selected against during chronic infection. As described by several studies [51] [27] [17], the shift from an opportunistic toward a lifelong persistent phenotype has a major impact in dampening the innate immune recognition and deteriorating the lung function in CF patients. This evolutionary scenario is similar to that of the genomes of other pathogens. Genetic loss-of-function mutations confer indeed enhanced fitness of the pathogen in a host-associated environment as shown for other pathogen [122] [123] [124].

This raises the question whether anti-virulence therapy could favor *P. aeruginosa* fitness to the CF lung leading to chronic infections. One possibility could be to target virulence genes only during acute phases of *P. aeruginosa* infection in CF patients, aiming to the eradication by the host immune system or to target virulence genes highly conserved during the progression of CF lung disease.

E.2 Host genetic background influences the response to *P. aeruginosa* infection

An increasing number of papers reports that the host genetic background significantly modulates the outcome of infectious diseases highlighting that in most cases multiple complex genetic interactions may have a key role in controlling infection [125]. These findings reveal that pathogens and their associated phenotypes are not the only determinants of the corresponding infectious diseases. In the case of *P. aeruginosa* opportunistic infection, much influence on disease outcome has been mainly attributed to different bacterial phenotypes rather than to host genetic background. In the second part of my work thesis we selected nine inbred murine strains characterized by a wealth of genetic and phenotypic diversity and representing in a discrete part the

numerous hallmarks of the human population [98]. We show that different inbred murine strains are highly variable in their response to acute airway infection. During a time course analysis, a wide-range response to *P. aeruginosa* infection has been observed both in the survival rate and body weight change of different inbred murine strains. Most notably, deviant clinical phenotypes were observed being the A/J, 129S2/SvPasCRL and DBA/2J as the most susceptible while BALB/cAnNCrl and C3H/HeOuJ the most resistant mouse strains. Other murine strains (BALB/cJ, BALB/cByJ, C57BL/6J and C57BL/6NCrl) were found be relatively resistant. Furthermore, we demonstrate no significant behavioral differences among distinct sub-strains of C57BL/6 from Jackson (C57BL/6J) or Charles River (C57BL/6NCrl). As reported in other studies, recent observations suggest that different variants of C57BL/6 mice are similar when monitored for acute mortality but have a different susceptibility to *P. aeruginosa* infection in terms of chronic persistence [51]. When distinct sub-strains of BALB/c from Jackson (BALBc/J and BALB/cByJ) or Charles River (BALB/cAnNCrl) were compared, we did not find any obvious differences. In this context, it is interesting to note that the widely used CB57BL/6 mice were not the most susceptible compared with other inbred strains. CB57BL/6 is also the background of *Cftr*-ko mice and it is known that these mice do not mimic human CF lung disease as in humans [126]. Our results open the question whether other mouse genetic background may better recapitulate the pulmonary abnormalities of CF patients.

Some of the inbred mouse strains used in this work were tested previously by others employing different model systems [106] [80]. Intraperitoneal *P. aeruginosa* infection of 16 different strains of inbred mice showed enhanced resistance for C3H mice and susceptibility for A.BY [106] [127]. Most strains, including DBA/2J, C57Bl6/J and BALB/c, used also in our work, showed no significant differences. In another work, 11 inbred mouse strains were compared using aerosol model of *P. aeruginosa* infection [128]. DBA/2 were the most susceptible while A/J, C3H/HeN, SWR/J and B10.D2/nSnJ were the most resistant when monitored for mortality. Using a chronic model of *P. aeruginosa* infection and evaluating mortality and bacterial load, a more restricted number of four inbred mouse strains were compared. BALB/c mice were found to be resistant, and DBA/2 mice were identified as the most susceptible strain while C57BL/6NHsd and A/JCr mice were found to be relatively susceptible [107]. Additional papers characterized inflammatory response directly comparing one resistant and one susceptible mice considering as resistant and susceptible BALB/c and C57BL/6 [109][110], BALB/c and DBA/2 [108], C3H/HeN and BALB/c respectively [129, 130]. Taken together our results and those of previous reports, it seems that a comprehensive classification of mouse inbred strains into categories according to their resistance or susceptibility is difficult to achieve As demonstrated for *P. aeruginosa* and other pathogens, the

differences in the disease outcome may be affected by the site of infection [131]; [132]. In the above mentioned works, different routes of administration have been used for *P. aeruginosa* infection including intraperitoneal [127], aerosol [133] or intratracheal (this work). Other factors have also been shown to influence the disease phenotype and, by interference, susceptibility or resistance. The size of the inoculum and the strain of the pathogen used seem to be particularly important. Different *P. aeruginosa* bacterial strains have been used including laboratory PAO1 [133] or clinical strains PA-103, Fisher type 4 [127], and AA2 in this work. These results reveal a previously unrevealed level of complexity and show that conclusions regarding disease susceptibility induced by *P. aeruginosa* infection may be highly dependent on the experimental model that is used. Based on this body of evidence, it seems that a comprehensive classification of mouse inbred strains into categories according to their resistance or susceptibility is difficult to achieve.

Mouse inbred strains are the starting point from which to explore causal phenotype-genotype relationships, including the identification of cell-mediate immune response and gene mapping. In this work, the susceptible A/J mice and the resistant C3H/HeOuJ have been used to gain deeper insight into the cellular and molecular factors that may contribute to different disease pathogenesis. High bacterial replication and inadequate immune-response was observed for A/J mice suggesting that susceptible mice are inefficient to keep in check the bacterial infection. In other words, A/J mice do not mount a proper, early immune defence leading to a permissive environment for bacterial replication and spreading from the airways to other organs and ultimately to a fast disease progression. The lack of the inflammatory response in A/J mice may not be attributable to deficient cytokines or chemokines response, rather mice showed an excessive or uncontrolled release of pro-inflammatory cytokines suggesting an ongoing cytokine storm. Previous studies have demonstrated that A/J mice are known to carry a loss-of-function mutation at the C5 locus, which is implicated in mobilizing inflammatory cells, in particular neutrophils, critical for host defense against infection [134]. Although this deficiency has been widely demonstrated to account for the susceptibility of A/J mice to several pathogens, such as *Candida albicans* [135] and *Mycobacterium tuberculosis* [136], we hypothesize that the C5 deficiency might not be completely responsible for *P. aeruginosa* susceptibility, since inbred strains deficient (A/J and DBA/2) or non-deficient for C5 (129S2/SvPasCRL) are equally susceptible to *P. aeruginosa* infection In contrast to susceptible A/J mice, resistant C3H/HeOuJ mice mount a faster and consistent immune-response that is able to efficiently control bacterial replication. In more detail, C3H/HeOuJ mice exhibited a prompter recruitment of inflammatory cells, mainly PMN, to the site of infection than did mice of the A/J strain. A balanced level of cytokines or chemokines has been observed in C3H/HeOuJ mice.

Furthermore, after a strong PMNs response of C3H/HeOuJ resistant mice, a progressive increase in macrophages involvement and BALT-like structure activation was observed. This BALT-like structure activation, instead, was substantially never observed in A/J susceptible strain. These differences clearly indicates that, in this model of infection, the two mouse strains react differently in modulation of inflammatory response and probably in the way of the antigen presentation to the lymphocytes of BALT. These cells may play a role in mounting a cell-mediated response to determine effective control or bacterial clearance.

Other papers on inbred mice analysed the inflammatory response in susceptible and resistant strains showing controversial results about the biological significance of the early inflammatory response in modulating the course and outcome of *P. aeruginosa* infection. In agreement with our results, Morissette and co-workers reported a rapid influx of polymorphonuclear cells (PMN), which was shortly followed by an efficient clearance of bacteria in BALB/c resistant mice, while DBA/2 susceptible mice had a delay in both the inflammatory response and the initiation of bacterial clearance [83, 108]However, other papers report an accumulation of inflammatory cells in susceptible rather than resistant mice. An exaggerated inflammatory response dominated by PMN correlates with susceptibility to infection in C57Bl/6 mice, whilst a modest inflammatory response dominated with macrophages correlated with resistance in BALB/c mice [110] [109]. Nevertheless, it should be expected that differences in the experimental model used (acute versus chronic) may affect several physiological parameters, adding complexity to the overall picture of the *P. aeruginosa*/host interaction.

Taken together, our results showed that survival to *P. aeruginosa* infection is clearly affected by genetic background. Comparative analysis of the cell-mediated immunity to *P. aeruginosa* infection in resistant and susceptible strains has been used in determining the key players of a successful versus an unsuccessful response to infection. During this early phase of infection, a prompt inflammatory response in the airways provides a biological advantage in creating a non-permissive environment for *P. aeruginosa* replication and restraining the spread to other organs. Thus, we speculate that host genetic make-up may have a role in the reduction of cell-mediated immunity that could be critical in the control of *P. aeruginosa* infection.

So far, we identified mouse strains presenting deviant clinical and immunological phenotypes which are suitable for genetic analyses. F2 mouse lines generated from A/J susceptible and C3H/HeOuJ resistant mouse strain are under evaluation to identify the genes controlling the host lung response to *P. aeruginosa* infection by Quantitative traits loci mapping. Identification of these genetic factors will provide insight into the key molecular processes that control host/pathogen interactions and might reveal novel targets for therapeutic interventions.

Conclusion

As suggested by our results, the complexity of host- pathogen interaction is strongly dependent upon two principal factors: the pathogen with its multifactorial and combinatorial virulence, and the host with its specific genetic background. Actually, the availability of bacteria and mouse complete genome sequence lead to a major boost in our understanding of host-pathogen interaction. In addition, a combined genetic strategy, applied both to the pathogen (e.g. random transposon mutation) and to the host (forward genetic analysis of susceptibility to infection), will shed considerable light on bacterial and host key factors involved in this interaction, favouring new therapeutic approaches in patients at risk of *P. aeruginosa* infections.

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Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis Airways Influences Virulence of *Staphylococcus aureus In Vitro* and Murine Models of Co-Infection

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Abstract

Cystic fibrosis (CF) airways disease represents an example of polymicrobial infection whereby different bacterial species can interact and influence each other. In CF patients *Staphylococcus aureus* is often the initial pathogen colonizing the lungs during childhood, while *Pseudomonas aeruginosa* is the predominant pathogen isolated in adolescents and adults. During chronic infection, *P. aeruginosa* undergoes adaptation to cope with antimicrobial therapy, host response and co-infecting pathogens. However, *S. aureus* and *P. aeruginosa* often co-exist in the same niche influencing the CF pathogenesis. The goal of this study was to investigate the reciprocal interaction of *P. aeruginosa* and *S. aureus* and understand the influence of *P. aeruginosa* adaptation to the CF lung in order to gain important insight on the interplay occurring between the two main pathogens of CF airways, which is still largely unknown. *P. aeruginosa* reference strains and eight lineages of clinical strains, including early and late clonal isolates from different patients with CF, were tested for growth inhibition of *S. aureus*. Next, *P. aeruginosa/S. aureus* competition was investigated in planktonic co-culture, biofilm, and mouse pneumonia model. *P. aeruginosa* reference and early strains, isolated at the onset of chronic infection, outcompeted *S. aureus in vitro* and *in vivo* models of co-infection. On the contrary, our results indicated a reduced capacity to outcompete *S. aureus* of *P. aeruginosa* patho-adaptive strains, isolated after several years of chronic infection and carrying several phenotypic changes temporally associated with CF lung adaptation. Our findings provide relevant information with respect to interspecies interaction and disease progression in CF.

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Introduction

Chronic airway infections and inflammation cause progressive lung disease and are the leading causes of mortality in patients with cystic fibrosis (CF) [1]. CF disease is characterized by the accumulation of secretion in the lungs and by a decreased mucociliary clearance that lead to an impaired ability to defeat bacterial infections. The viscous CF lung secretions provide an environment that protects bacteria from the assault of antibiotics and immune cells, thus favoring colonization and persistence. CF patients have a unique set of bacterial pathogens that are frequently acquired in an age dependent sequence [2]. The most frequently cultured organisms from the respiratory tract of young children are *Staphylococcus aureus* and non-typeable *Haemophilus influenzae*. Later, as the patient ages, infection progresses to involve opportunistic pathogens such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*.

It is now becoming clear that the different bacteria coexisting in CF airways have a mutual interaction and contribute to the pathogenesis of the disease [3,4]. In a context that involves a complex polymicrobial community a single-species microbial analysis could be inadequate, as different microbes within the community can interact each other and the resulting infection pathogenesis differs from that in infections caused by the component species individually [3,5]. Chronic bacterial infections associated with CF lung disease have been studied by a range of culture-independent profiling methodologies [6 12], and each approach has revealed greater microbial diversity than was previously recognized. Overall, the results of these studies suggest that the polymicrobial nature of CF infections could play a key role in driving disease and response to therapy and, in turn, significantly impact upon clinical outcomes [1,7,13]. Nevertheless, very little is known about the role of interspecies interactions in the pathogenesis of the CF lung disease [14,15].

The Gram-positive bacterium *S. aureus* is the pathogen most commonly isolated in nasopharyngeal samples from young children with CF, and in the preantibiotic era, many CF patients succumbed to *S. aureus* infection [16]. Recent data demonstrate an increase in *S. aureus* infections in the CF population, not only in the US but also in Europe, with methicillin-resistant *S. aureus* (MRSA)

strains being on the rise [17,18], reflecting the overall increase in prevalence and epidemiologic changes in the general population [19,20].

Of the multiple opportunistic bacteria that may infect CF patients, the Gram-negative bacterium *P. aeruginosa* is considered to be the most significant as it has clearly been linked to worsening of the pulmonary status [21]. Despite intensive antibiotic treatments, *P. aeruginosa* infections are difficult to eradicate [22]. The antibiotic treatment may favor the emergence of antimicrobial drug resistance. One of the most striking characteristics of *P. aeruginosa* chronic lung infection in CF patients is indeed the co-existence of multiple phenotypes that are highly resistant to any chemotherapy treatment [23].

Although *S. aureus* colonization/infection usually precedes chronic colonization of the respiratory tract by *P. aeruginosa*, it continues into adulthood, when 51% of patients become culture positive for *S. aureus* [24]. Both organisms are commonly co-isolated from CF respiratory cultures and it has been shown that risk factors for initial *P. aeruginosa* airway infection in patients with CF include *S. aureus* pre-colonization [25,26]. In addition, both species are able to shift between a planktonic (free-living) life style to surface-attached communities known as biofilms during chronic infections. In human diseases including CF, biofilm-related infections are directly correlated with dramatic increases in antibiotic resistance [27,28,29].

In this study, we aimed to explore the interactions between *S. aureus* and *P. aeruginosa* by using *in vitro* and murine models of pneumonia. During chronic infection, *P. aeruginosa* undergoes numerous selective pressures ranging from antibiotic treatments, host immune response and interactions with other microorganisms leading to the development of patho-adaptive lineages. The adaptation of *P. aeruginosa* to the CF niche selects for clones with reduced virulence in multi-hosts models [23,30]. We focused our attention on the reciprocal influence of *P. aeruginosa* and *S. aureus* and on understanding how *P. aeruginosa* adaptation to the CF lung may interfere with *S. aureus* interaction. Using a collection of longitudinal strains isolated from CF patients, we showed that *P. aeruginosa* strains out-competed *S. aureus*. This effect was associated with *P. aeruginosa* early strains, which in acute infection present higher virulence. On the contrary, *P. aeruginosa* late adapted strains showed reduced or abolished capacity to outcompete *S. aureus*. This work provides key results on lung pathogenicity caused by multi-bacterial infection.

Results

P. aeruginosa early and late clonal variants differently influence growth of *S. aureus*

Eight lineages of *P. aeruginosa* strains, including 12 early (early group) and 12 late (late group) clonal isolates from different patients with CF were tested for growth inhibition of *S. aureus* Newman and SH1000 strains on agar surfaces [25]. In particular, late *P. aeruginosa* strains selected for this study were collected over a period of 16.3 years and carried several patho-adaptive traits, including mucoid and hypermutable phenotypes (Table 1) as reported previously [23,31]. In addition, PAO1 and PA14 *P. aeruginosa* reference strains, which show phenotypic traits characteristic of early isolates [23], were also included.

As shown in Table 1, growth of *S. aureus* Newman and SH1000 strains was inhibited by PA14 and PAO1 *P. aeruginosa* reference strains and by 100% (12/12) and 91.6% (11/12) of *P. aeruginosa* early strains respectively in co-culture. The only exception was the strain KK1 which was previously described as different in terms of virulence potential from KK2, isolated at the same time point [23]. The strength of inhibition of *S. aureus* in some cases differed within clonal lineages (TR1 vs TR2; MF1 vs MF2; KK1 vs KK2).

Differently from *P. aeruginosa* early strains, 58.4% (7/12) of the late strains had no effect on growth of *S. aureus* Newman and SH1000 strains. These *P. aeruginosa* strains belonged to six different lineages (NN, BT, AA, TR, MF, KK) indicating presence of at least one *P. aeruginosa* strain unable to inhibit *S. aureus* growth in the majority of CF patients (75%: 6/8). The other two *P. aeruginosa* lineages (SG and BST) (25%: 2/8) inhibited *S. aureus* growth although to a lesser extent when compared to early strains. Late *P. aeruginosa* strains within the same lineage also differed with regard to the strength of *S. aureus* growth inhibition (SG57 vs SG58; BT72 vs BT73; TR66 vs TR67), indicating a diversification of the bacterial population during chronic infection as demonstrated for other virulence traits [23,32]. The average inhibition halo of late group was 11.6 mm versus Newman and 11.3 mm versus SH1000, while the average inhibition halo of early group was 18.3 mm versus Newman and 17.4 mm versus SH1000. Late group showed a statistically significant effect in reducing levels of inhibition (regression parameter = −6.76 versus Newman and regression parameter = −6.24 versus SH1000) with $p<0.01$ for both settings. This data indicated that, as a group, late *P. aeruginosa* strains differ significantly from early strains in their capacity to inhibit *S. aureus* growth, suggesting a trend of *P. aeruginosa* patho-adaptive variants to influence the growth of *S. aureus*.

On the contrary *S. aureus* did not exert any effect on the growth of *P. aeruginosa* (Table S1).

Competition between *S. aureus* and *P. aeruginosa* in planktonic co-cultures

Next, we investigated the interactions between *S. aureus* and *P. aeruginosa* in planktonic growth by comparing the growth kinetics of the two organisms in co-culture to those obtained in pure culture. One reference *P. aeruginosa* strain PA14 and a pair of sequential strains from patient AA were selected. Figure 1A shows the growth curves of the reference *S. aureus* Newman and *P. aeruginosa* PA14 strains in single and dual cultures. PA14 maintained the same growth rate in pure culture and in co-culture, and had a significant negative effect on the overall trend of the growth of Newman ($p<0.001$). In order to have a clear comprehension of the differences in growth between *S. aureus* and *P. aeruginosa*, we calculated the Competition Index (CI), that allows to compare the differences in growth curve of mixed cultures, and the CI-like index, the Relative Increase Ratio (RIR), that compares the growth curves of the two species in pure culture (see Materials and Methods). As shown in Figure 1B, the CI of PA14 versus Newman was significantly different from the RIR in late exponential phase (12 h, $p<0.001$) and stationary phase (24 h, $p<0.001$) of growth, suggesting an inhibitory effect of *P. aeruginosa* on *S. aureus*.

Next, we explored the effect of *P. aeruginosa* strains isolated at the onset of chronic colonization (early strains) or several years after acquisition (late strains) from CF patients on growth of *S. aureus*. A pair of well characterized *P. aeruginosa* clonal strains isolated from CF patient were selected: the AA2 early strain and AA43 late adapted strain carrying several phenotypic changes in virulence factor production, and patho-adaptive mutations within the genome temporally associated with CF lung infection [23,30,33]. The growth of Newman was significantly inhibited by the presence of the early AA2 strain ($p<0.001$), while AA2 strain was not affected from the presence of *S. aureus* (Figure 1C). The CI of AA2 versus Newman was significantly higher than the RIR in late exponential phase (8 h, $p<0.05$ and 12 h, $p<0.01$, Figure 1D) and in stationary phase (24 h, $p<0.01$). On the other hand, Newman and the late strain AA43 interfered each other in co-culture,

Table 1. *In vitro* growth inhibition of *S. aureus* and *P. aeruginosa*.

P. aeruginosa spot	*S. aureus* lawn (Newman) (inhibition halo)	*S. aureus* lawn (SH1000) (inhibition halo)
PAO1	strong (24.5 mm)	strong (20 mm)
PA14	strong (22 mm)	strong (19 mm)
SG1	strong (23.5 mm)	strong (22.5 mm)
SG57*	strong (23 mm)	strong (20.5 mm)
SG58*	weak (14.5 mm)	weak (15 mm)
NN2	weak (15 mm)	weak (14 mm)
NN83#*	no (9 mm)	no (9 mm)
BT1#	weak (15 mm)	weak (14.5 mm)
BT2	weak (15 mm)	weak (15 mm)
BT72*	no (9 mm)	no (9 mm)
BT73*	weak (13.5 mm)	weak (13 mm)
AA2	very strong (27 mm)	strong (20.5 mm)
AA43*	no (9 mm)	no (9 mm)
TR1	weak (13.5 mm)	weak (13.5 mm)
TR2	strong (20 mm)	strong (20 mm)
TR66*	weak (11.5 mm)	weak (11.5 mm)
TR67*	no (9 mm)	no (9 mm)
MF1	weak (15 mm)	strong (21 mm)
MF2#	strong (21 mm)	strong (18.5 mm)
MF51*	no (9 mm)	no (9 mm)
KK1	weak (12 mm)	no (9 mm)
KK2	very strong (27 mm)	very strong (26.5 mm)
KK71*	no (9 mm)	no (9 mm)
KK72*	no (9 mm)	no (9 mm)
BST2	weak (15 mm)	weak (14.5 mm)
BST44#*	weak (14.5 mm)	weak (12.5 mm)

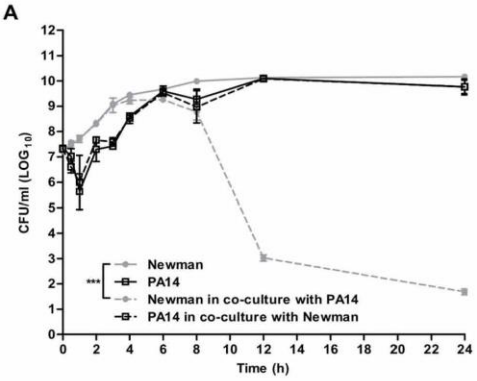
Twenty-four *P. aeruginosa* isolates were collected from eight individuals with CF (SG, NN, BT, AA, TR, MF, KK, BST) at the onset of chronic colonization (numbered 1-2) or after 4.5–16.3 years of colonization (numbered 43-83). PAO1 and PA14 were included as reference strains. 5 µl spots of *P. aeruginosa* overnight cultures, normalized to 0.5 OD, were added to *S. aureus* lawn (normalized to 0.5 OD) on Mueller-Hinton agar and incubated overnight at 37°C. The table summarizes the results obtained: "weak inhibition" indicates an inhibition halo ≤15 mm; "strong inhibition" indicates an inhibition halo >15 mm and ≤25 mm; "very strong inhibition" indicates an inhibition halo >25 mm; "no inhibition" indicates absence of inhibition halo (9 mm is the diameter of the *P. aeruginosa* spot).
* Indicates mucoid phenotype.
#Indicates hypermutable phenotype. For statistical analysis see "Results".
doi:10.1371/journal.pone.0089614.t001

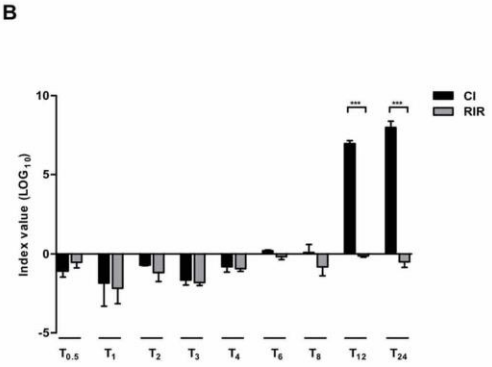
slightly but significantly reducing their growth rate compared to pure culture ($p<0.001$, Figure 1E). It is worth noting that AA43 inhibited the growth of Newman to a lower extent compared to AA2: while AA2 determined a reduction of 3, 5 and 6 log at 8, 12 and 24 h respectively, AA43 determined a reduction of less than 1 log at the same time points (Figure 1C and E). Being the competition reciprocal between the two species and considering their different growth rate in pure culture, the CI did not differ from the RIR (Figure 1F). Similar results were obtained using the same isolates of *P. aeruginosa*, AA2 and AA43, in co-culture with the reference *S. aureus* SH1000 (Figure S1) strengthening the results obtained with Newman. Taken together these data indicate that *P. aeruginosa* strains, including reference or those isolated at the early stage of chronic infection, can outcompete *S. aureus* in planktonic cultures. On the other hand, *P. aeruginosa* patho-adaptive strains lose this capacity over time.

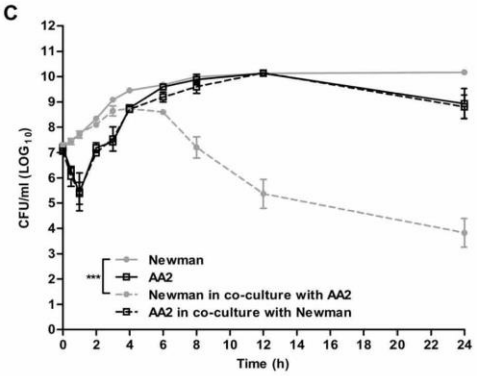
S. aureus and *P. aeruginosa* interaction in biofilm

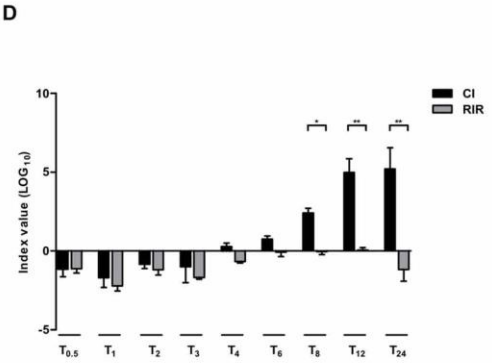
In order to understand if the reciprocal interaction among the two species could affect their capacity to produce biofilm, we quantified the biofilm biomass of individually cultured or co-cultured at a ratio 1:1 *S. aureus* and *P. aeruginosa* by staining with crystal violet. As shown in Figure 2, the results obtained from co-cultured pair of strains formed by Newman and PA14 revealed significantly lower level of biomass compared to Newman only (Newman vs Newman+PA14 $p<0.01$), but similar to that corresponding to PA14 alone. This data suggests an inhibitory effect exerted by *P. aeruginosa* on *S. aureus* biofilm formation. For clinical *P. aeruginosa* strains, while the OD value detected in the mixed biofilm formed by Newman and AA2 was not significantly different from both Newman and AA2 individually cultured, the OD value associated to the mixed biofilm formed by Newman and AA43 revealed significantly lower levels of biomass compared to both Newman (Newman vs Newman+AA43 $p<0.001$) and AA43 (AA43 vs Newman+AA43 $p<0.001$) individually cultured. This finding suggests a reciprocal interference of the two species, confirming the results of batch co-culture experiments.

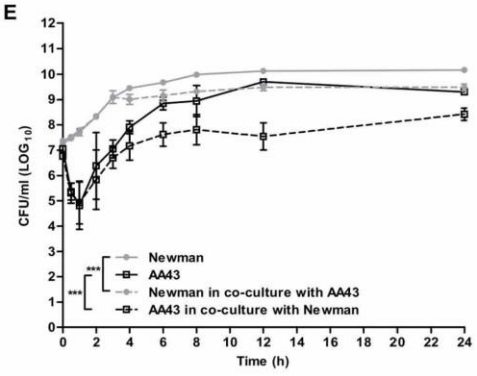
We also determined the amount of viable bacteria of each species in both planktonic and sessile fractions in single and dual cultures. In co-culture, we noticed that all strains of *P. aeruginosa*


A
CFU/ml (LOG10)
Time (h)
Newman
PA14
Newman in co-culture with PA14
PA14 in co-culture with Newman
***


B
Index value (LOG10)
CI
RIR
***
T0.5 T1 T2 T3 T4 T6 T8 T12 T24


C
CFU/ml (LOG10)
Time (h)
Newman
AA2
Newman in co-culture with AA2
AA2 in co-culture with Newman
***


D
Index value (LOG10)
CI
RIR
*
**
**
T0.5 T1 T2 T3 T4 T6 T8 T12 T24


E
CFU/ml (LOG10)
Time (h)
Newman
AA43
Newman in co-culture with AA43
AA43 in co-culture with Newman
***
***

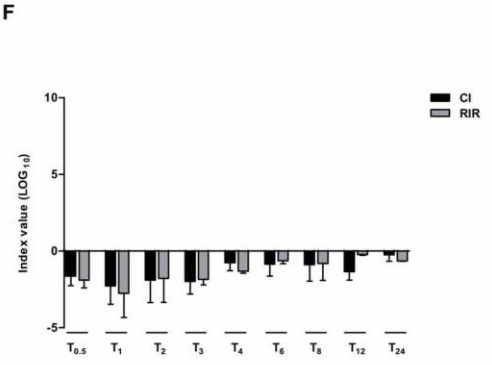

F
Index value (LOG10)
CI
RIR
T0.5 T1 T2 T3 T4 T6 T8 T12 T24

Figure 1. Single and dual species batch growth curves and competition index values. *S. aureus* strain (Newman) and *P. aeruginosa* strains (PA14 and two clinical early and late isolates from a CF patient AA2 and AA43) were grown for 24 hours in BHI in single culture and in co-culture after inoculation at equal ratio from mid-exponential phase pure cultures. Growth rate was monitored by colony count after plating on selective media for both species. Results are represented as the mean of values obtained from three independent experiments. The error bars indicate the standard deviations. A nonlinear mixed-effect model was fitted, using a four-parameters logistic regression function. Panel A: growth curves of Newman in pure culture and in co-culture with PA14; Panel B: Competition index (CI) and Relative Increase Ratio (RIR) calculated from single and dual cultures of Newman and PA14; Panel C: growth curves of Newman in pure culture and in co-culture with AA2; Panel D: CI and RIR calculated from single and dual cultures of Newman and AA2; Panel E: growth curves of Newman in pure culture and in co-culture with AA43; Panel F: CI and RIR calculated from single and dual cultures of Newman and AA43. Each value represents the mean of CI and RIR values from three independent experiments and the bars indicate standard deviation. Statistically significant differences in Student's t test and in nonlinear mixed-effect model are indicated by symbols when present: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.
doi:10.1371/journal.pone.0089614.g001

tested determined a reduction of the number of both sessile and planktonic Newman cells ($p<0.001$) (Figure 3A). In particular, the bacterial load of Newman in sessile fraction, when co-cultured with clinical early *P. aeruginosa* AA2, decreased of five log compared to pure culture, while the clonal late strain AA43 caused a lower (two log) reduction. A similar effect was observed also in planktonic fraction (Figure 3A) in agreement with batch co-culture data.

On the contrary, the presence of Newman had no effect on PA14 and AA2 growth in both planktonic and biofilm fractions, while it moderately inhibited the attachment to polystyrene and biofilm formation of the late *P. aeruginosa* strain AA43, confirming a reciprocal interaction between Newman and AA43 (Figure 3B, $p<0.001$).

Figure 4 shows the percentage of planktonic and sessile cells of the two species in single and dual cultures. While Newman in pure culture presented the highest percentage of sessile cells, in dual culture was negatively affected by the presence of PA14 and AA2, and the biofilm composition of the co-culture reflected that of *P. aeruginosa* in pure culture (Figure 4A and 4B). A reciprocal influence was evident only for the pair represented by Newman and the late strain AA43 (Figure 4C). It is worth noting that in single species biofilm, the mucoid AA43 strain, even if apparently displaying a lower biofilm biomass compared to AA2 after staining with crystal violet, presented a higher percentage of sessile cells compared to AA2 (5.7% vs 3.4% respectively).

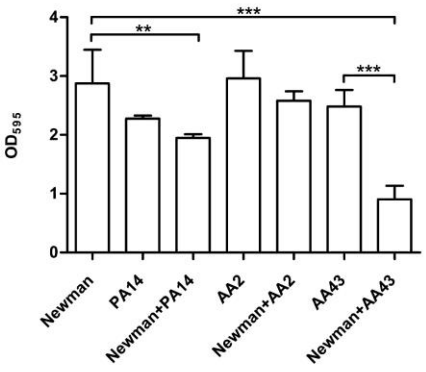


Figure 2. Biofilm formation by *S. aureus* and *P. aeruginosa* strains in single and dual cultures. Bacteria were grown overnight in 96-well flat-bottom microtiter plates in NB medium at 37°C either individually cultured or co-cultured at a 1:1 ratio. Biofilm biomass was quantified by staining with crystal violet and absorbance measurements at OD 595 nm. The values represent the means of three independent experiments, and the bars indicate standard deviation. Statistically significant differences in Student's t test are indicated by symbols when present: **: $p<0.01$; ***: $p<0.001$.
doi:10.1371/journal.pone.0089614.g002

Competition between *P. aeruginosa* and *S. aureus* in a mouse model of acute lung infection

To test whether the observed differences in planktonic growth and biofilm formation *in vitro* would be relevant *in vivo*, a mouse model of acute pneumonia was used. Thus, we set up *in vivo* competition between *P. aeruginosa* and *S. aureus* in C57Bl/6NCrlBR mice challenged with 1×10^6 CFU of *S. aureus* and *P. aeruginosa* strains mixed together at a 1:1 ratio. Eighteen hours after infection, murine lungs were homogenized and plated. Differential CFU counting was performed to calculate the CI. Results show that *P. aeruginosa* reference strain PA14 and the early isolate AA2 outcompeted *S. aureus* strain Newman, as the CI, being significantly different from 1, indicated a competitive advantage of *P. aeruginosa* over *S. aureus* (PA14/Newman average CI = 5.0, $p<0.01$; AA2/Newman average CI = 3.3, $p<0.05$). Different results were obtained for the *P. aeruginosa* late isolate AA43 and Newman as the CI 18 hours after challenge was not significantly different from 1 (AA43/Newman CI = 0.9), indicating no competition in this case (Figure 5 and Table 2).

Discussion

The goal of this study was to investigate the influence of *P. aeruginosa* adaptation to the CF lung on interaction with *S. aureus* in co-culture, during biofilm formation and mouse lung infection, in order to gain important insight on the interplay occurring between the two main pathogens of CF airways, which is still largely unknown. For this purpose, we used a panel of deeply genetically and phenotypically characterized *P. aeruginosa* clonal strains isolated from CF patients at different time points during CF chronic lung infection [33,34].

We evaluated the inhibitory effect of eight *P. aeruginosa* lineages on *S. aureus*, including strains isolated both at early and late stage of chronic infection. A negative effect on *S. aureus* growth significantly associated with early-infecting *P. aeruginosa* strains was observed, while clonal late-infecting *P. aeruginosa* strains presented a significantly reduced or abolished virulence when co-cultivated with *S. aureus*. During chronic infection, *P. aeruginosa* undergoes adaptation to the CF lung, leading to patho-adaptive lineages that differ genotypically and phenotypically from the originally infecting strain. Such microevolution usually determines loss of motility, acquisition of mucoidy, antibiotic resistance and loss-of-function mutations in virulence genes, suggesting attenuation of virulence for CF adapted strains [30,35,36,37]. Here we demonstrated for the first time that *P. aeruginosa* virulence traits affect also the interaction with other CF-related pathogen as *S. aureus*. As described for other traits, intra-clonal variation was observed both in clonal *P. aeruginosa* early strains and late strains isolated at the same time from the CF patients. One of the most striking

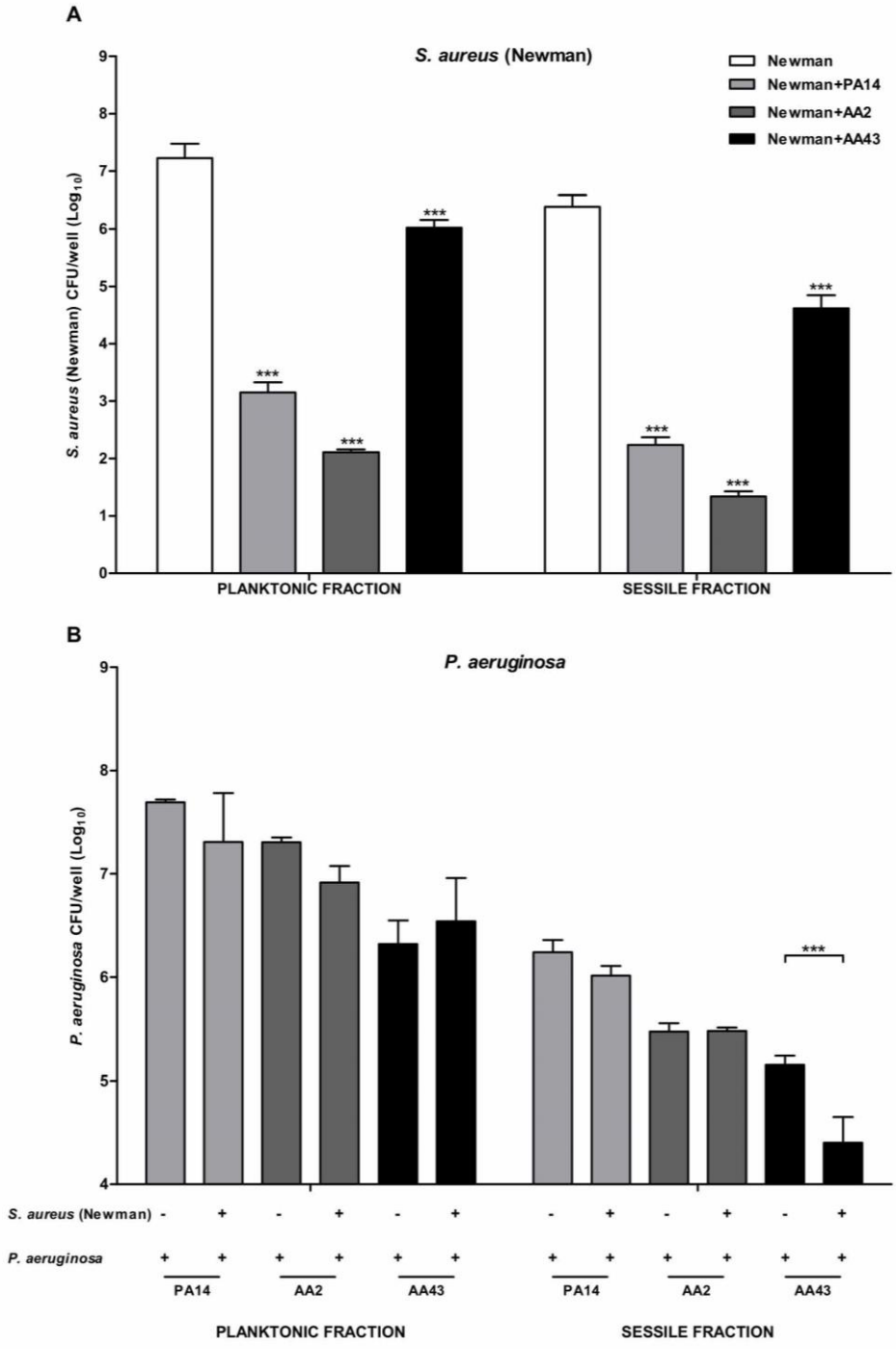

A
S. aureus (Newman)
Newman
Newman+PA14
Newman+AA2
Newman+AA43
S. aureus (Newman) CFU/well (Log10)
***
PLANKTONIC FRACTION
SESSILE FRACTION
B
P. aeruginosa
P. aeruginosa CFU/well (Log10)
S. aureus (Newman)
P. aeruginosa
PA14
AA2
AA43
PLANKTONIC FRACTION
SESSILE FRACTION

Figure 3. *S. aureus* and *P. aeruginosa* planktonic and sessile cells in single and dual cultures. Bacteria were grown overnight in 96-well flat-bottom microtiter plates in NB medium at 37°C either individually cultured or co-cultured at a 1:1 ratio. CFU counts were determined in both planktonic and sessile fractions. Panel A: planktonic (left) and sessile (right) cells of *S. aureus* strain Newman in pure culture and in co-culture with *P. aeruginosa* strains PA14, AA2 and AA43. Statistically significant differences are referred to Newman in pure culture. Panel B: planktonic (left) and sessile (right) cells of *P. aeruginosa* strains PA14, AA2 and AA43 in pure culture and in co-culture with *S. aureus* strain Newman. The values represent the means of three independent experiments, and the bars indicate standard deviation. Statistically significant differences in non-parametric Mann–Whitney test are indicated by symbols when present: **: $p<0.01$; ***: $p<0.001$.
doi:10.1371/journal.pone.0089614.g003

characteristics of *P. aeruginosa* chronic lung infection in CF patients is the intense diversification of the bacterial population, leading to the co-existence of multiple phenotypes that may colonize different airways niches. Thus, the intra-clonal variation that we have observed is most likely the result of this process of genetic adaptation.

Under planktonic growth conditions, we have shown that both the reference *P. aeruginosa* strain PA14 and the clinical early strain AA2 strongly inhibited the growth of *S. aureus* during late logarithmic phase and stationary phase, without being influenced in their growth rate. Antagonism between microorganisms within a community could be attributed to simple competition for limited resources or to direct antagonistic effects [36]. There is evidence supporting antagonism between *P. aeruginosa* and *S. aureus*. Mashburn *et al.* demonstrated that *P. aeruginosa* can lyse *S. aureus* to use the iron released for its own growth [38]. Moreover, it has been reported that *S. aureus* is susceptible to an arsenal of respiratory inhibitors generated by *P. aeruginosa*, such as pyocyanin, hydrogen cyanide or alkyl-hydroxyquinoline N-oxides (HQNO), which are able to suppress the aerobic metabolism and growth of *S. aureus* [25,39]. Interestingly, the late *P. aeruginosa* strain AA43, clonal to AA2, inhibited the growth of *S. aureus* at a lower extent, compared to AA2 and was not able to outcompete it. Besides being less virulent, AA43 was also negatively affected by the presence of *S. aureus* as its growth rate was significantly slowed down by *S. aureus* cells.

Despite the increasing interest on the crucial role of biofilm in CF infections, interspecies interactions of different organisms in mixed species biofilms are still poorly understood [27]. Here we have shown that in co-culture biofilms all *P. aeruginosa* strains were able to outcompete *S. aureus* in both sessile and planktonic fractions and the composition of the population in mixed biofilms was determined by *P. aeruginosa*, albeit to different extent. Also under biofilm growth conditions, the clonal late *P. aeruginosa* strains AA43 presented a different behavior in the presence of *S. aureus* compared to the early AA2 strain. In single species biofilm, the mucoid AA43 strain, even if apparently displaying a lower biofilm biomass compared to AA2 after staining with crystal violet, presented a higher percentage of sessile cells compared to AA2. This difference in biofilm production reflects the well documented phenotypic changes occurring in *P. aeruginosa* during the establishment of chronic infection. Indeed, *P. aeruginosa* strains isolated from CF patients at early stage of chronic infection are generally non-encapsulated and express a variety of virulence factors, whereas *P. aeruginosa* isolates from late stage typically lack virulence factors and convert to a mucoid phenotype, associated with greater biofilm formation and resistance to phagocytosis [37]. In apparent contradiction, also the early strain AA2 was able to produce biofilm. This could be explained considering the complexity of the microbial interactions in the CF lung, the presence of a diverse community of *P. aeruginosa* strains, and the many factors contributing to the formation of the biofilm matrix of *P. aeruginosa*, besides alginate production. In addition not all adapted isolates are

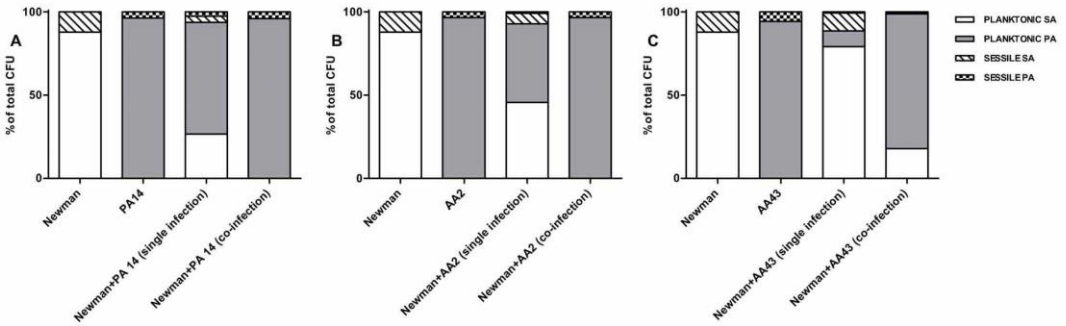


Figure 4. Percentage of planktonic and sessile cells in single and dual cultures. Bacteria were grown overnight in 96-well flat-bottom microtiter plates in NB medium at 37°C either individually cultured or co-cultured at a 1:1 ratio. CFU counts were determined in both planktonic and sessile fractions and the percentage of S. aureus and P. aeruginosa in the two fractions of single and dual cultures was calculated. Panel A: percentages of planktonic and sessile cells of Newman in single culture (first histogram), PA14 in single culture (second histogram), Newman and PA14 in ideal co-culture if the 2 species would not interfere each other (third histogram, percentages have been calculated considering the values of the first and second histograms), and Newman and PA14 in co-culture (fourth histogram). Panel B: percentages of planktonic and sessile cells of Newman in single culture (first histogram), AA2 in single culture (second histogram), Newman and AA2 in ideal co-culture if the 2 species would not interfere each other (third histogram, percentages have been calculated considering the values of the first and second histograms), and Newman and AA2 in co-culture (fourth histogram). Panel C: percentages of planktonic and sessile cells of Newman in single culture (first histogram), AA43 in single culture (second histogram), Newman and AA43 in ideal co-culture if the 2 species would not interfere each other (third histogram, percentages have been calculated considering the values of the first and second histograms), and Newman and AA43 in co-culture (fourth histogram). SA: S. aureus; PA: P. aeruginosa.
doi:10.1371/journal.pone.0089614.g004

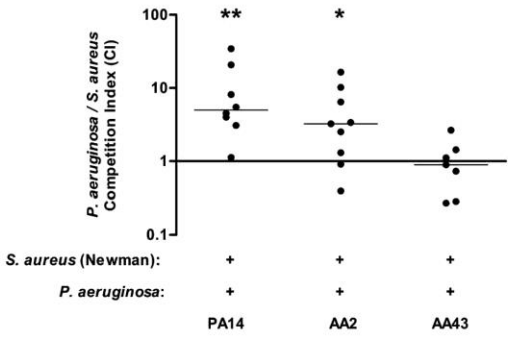


Figure 5. Competition between *P. aeruginosa* and *S. aureus* strains in a murine model of pneumoniae. Planktonic *S. aureus* strain Newman and *P. aeruginosa* clinical isolates AA2 and AA43 and reference strain PA14 were used to infect C57BL/6NCrlBR mice at a ratio of 1:1. After 18 hours of acute infection lungs homogenates were plated on selective plates to determine *S. aureus* and *P. aeruginosa* CFU. Each circle represents the CI for a single animal in each group. A CI value equal to 1 indicates equal competition of the two species; a CI value significantly <1 indicates a competitive advantage of *S. aureus* that outcompetes *P. aeruginosa*; a CI value significantly >1 indicates a competitive advantage of *P. aeruginosa* that outcompetes *S. aureus*. Wilcoxon signed rank test of the null hypothesis that the distribution of CI is symmetric about 1 was performed. Statistically significant differences are indicated by symbols when present: *: $p<0.05$; **: $p<0.01$. The data are pooled from two or three independent experiments.
doi:10.1371/journal.pone.0089614.g005

mucoid and also early not adapted strains could produce biofilm exploiting other biofilm matrix molecules [40]. In agreement with data obtained in planktonic co-cultures, AA2 strongly inhibited the growth of *S. aureus* in mixed biofilm, without being affected. Qazi *et al.* demonstrated that factors related to biofilm formation are down-regulated in *S. aureus* in response to *P. aeruginosa* presence, consistently with our results [41]. Compared to AA2, *P. aeruginosa* AA43 inhibited *S. aureus* growth at lower extent, determining a reduction of *S. aureus* CFU count of about 1 and 2 log in planktonic and sessile fractions respectively, when measured against *S. aureus* individual biofilm. Moreover, the capacity to produce biofilm of AA43 was negatively affected by the presence of *S. aureus*, confirming its attenuated virulence and susceptibility to competitor organism.

Although several studies using *in vitro* models demonstrated an inhibitory effect of *P. aeruginosa* on the growth of also highly virulent *S. aureus* strains such as USA 300, in line with our results [27,42,43], *in vivo* models show contradictory results [42,44]. We further investigated *S. aureus*/*P. aeruginosa* reciprocal interaction setting up a murine model of acute lung co-infection. In agreement with *in vitro* data, the reference strain PA14 and the early CF clinical isolate AA2, after 18 hours of co-infection, inhibited *S. aureus*, while the late CF clinical isolate AA43 did not outcompete *S. aureus*.

It is known that environmental and early clinical isolates of *P. aeruginosa* are equipped with a repertoire of virulence factors and, among them, also substances with anti-bacterial activity, these factors are selected against during the adaptation process to the CF airways environment. The results obtained in the acute pneumonia model, in which an early isolate is able to inhibit the growth of another pathogen, while its clonal adapted strain is no longer able to do so, strengthen the loss of anti-bacterial factors during adaptation.

Our data underline the importance of bacterial interactions in lung infection and in particular of the complexity of the interactions of different pathogens that coexist in the CF airways. However, given the genetic adaptation process of *P. aeruginosa* that leads to the selection of different patho-adaptive variants, descending from the initial infecting clone, further combinations of clonal lineages of early and late isolates should be tested to strengthen our *in vivo* data. Moreover, considering that the adaptation process during chronic infection involves also *S. aureus*, other experiments using clinical early and late *S. aureus* strains as well as adapted phenotypes such as small colony variants should be performed. Our results showing the influence of adaptation on the reciprocal interactions between *S. aureus* and *P. aeruginosa* deserve further investigations including the host response and the effect of environmental conditions, such as microaerobic and anaerobic

Table 2. Colonization of murine lungs with S. aureus and P. aeruginosa reference and clinical strains in competition experiments.

	PA14/Newman (n = 9[a])	AA2/Newman (n = 9[a])	AA43/Newman (n = 9[a])
Mortality, % (no. of dead/total mice)	0 (0/9)	0 (0/9)	0 (0/9)
Co-infected[b], % (no. of co-infected/surviving mice)	89 (8/9)	100 (9/9)	78 (7/9)
***P. aeruginosa* infected[c], % (no. of infected/surviving mice)**	100 (9/9)	100 (9/9)	78 (7/9)
***S. aureus* infected[d], % (no. of infected/surviving mice)**	89 (8/9)	100 (9/9)	78 (7/9)
Total cfu/lung[e]	3.3×10^4	6.7×10^3	5.8×10^3
***P. aeruginosa* cfu/lung[e]**	2.9×10^4	4.5×10^3	2.7×10^3
***S. aureus* cfu/lung[e]**	4.2×10^3	2.2×10^3	3.1×10^3
CI[f]	5.0	3.3	0.9

[a]Pooled mice, analyzed in two independent experiments.
[b]Co-infected mice, surviving after 18 hours from challenge.
[c]Number of pooled mice infected with *P. aeruginosa* after 18 hours.
[d]Number of pooled mice infected with *S. aureus* after 18 hours.
[e]Median values are reported.
[f]Competition Index.
doi:10.1371/journal.pone.0089614.t002

conditions, on pathogens interactions, using both *in vitro* and *in vivo* models of chronic infection that better mirror the progression of CF lung disease.

Materials and Methods

Animals and ethics statement

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC, Number 444) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals. All efforts were made to minimize the number of animals used and their suffering.

Research on *P. aeruginosa* bacterial isolates from the individuals with CF has been approved by the responsible physician at the CF center at Hannover Medical School, Germany. All patients gave informed consent before the sample collection. Approval for storing of biological materials was obtained by the Hannover Medical School, Germany.

Bacterial strains

For *S. aureus*, Newman and SH1000 reference strains, were used in the study [45,46]. Two *P. aeruginosa* reference strains, PA14 [47] and PAO1 [48], and 8 clonal lineages of *P. aeruginosa* clinical strains from CF patients (AA, SG, NN, BT, TR, MF, KK, BST), including strains isolated at the onset of chronic colonization (early: AA2, SG1, NN2, BT1, BT2, TR1, TR2, MF1, MF2, KK1, KK2, BST2) or several years after acquisition and before patient's death (late: AA43, SG57, SG58, NN83, BT72, BT73, TR66, TR67, MF51, KK71, KK72, BST44) were used in this study [23]. Clonality of strains, assessed by Pulsed Field Gel Electrophoresis and multiple phenotypic traits, including motility, mucoid phenotype, *LasR* phenotype, and pyocyanin secretion, have been determined and previously reported [23,32].

S. aureus growth inhibition on agar surface

S. aureus cultures (Newman and SH1000) grown overnight in Luria-Bertani broth (LB, Difco™) were normalized to 0.5 OD_{600} and uniformly spread on Mueller-Hinton agar plate (MH, Difco™) by using a cotton swab. Then 5 µl of *P. aeruginosa* culture, grown overnight in LB broth and normalized to 0.5 OD_{600}, were added to the *S. aureus* lawn followed by incubation overnight at 37°C [25]. The same procedure was performed spotting *S. aureus* culture on *P. aeruginosa* lawn. The following *P. aeruginosa* clonal lineages, including early and late clinical strains, were tested: AA, SG, NN, BT, TR, MF, KK and BST (for details see paragraph "Bacterial strains"). As *P. aeruginosa* reference strains we used PA14 and PAO1. The inhibition score was defined as follows: "no inhibition" when no halo was observed around the spot of *P. aeruginosa* that measures 9 mm; "weak inhibition" indicated an inhibition halo ≤15 mm; "strong inhibition" indicated an inhibition halo >15 mm and ≤25 mm; "very strong inhibition" indicated an inhibition halo >25 mm. The choice for inhibition strength ranges was based on preliminary assays performed using the lawn of about 30 *S. aureus* strains (including both reference and clinical strains of different origin) and spotting about 60 *P. aeruginosa* strains (both reference and clinical strains) on the different lawns.

Planktonic mono-culture and co-culture growth curves

All growth curves were performed in 30 ml of nutrient-rich not selective medium, Brain-Heart Infusion broth (BHI, Difco™), at 37°C with shaking (180 rpm). The following strains were tested: *S. aureus* (Newman, SH1000), *P. aeruginosa* (PA14, AA2 and AA43). Strains were grown overnight in BHI and subcultured in fresh medium for 2.5 hours to reach the mid-exponential phase of growth. Bacteria were centrifuged, pellet was resuspended in fresh medium and the OD_{600} was measured to adjust the concentration of bacteria. For co-cultures each pair of *S. aureus* and *P. aeruginosa* strains were inoculated at equal ratio (1 OD_{600}, optical density) from mid-exponential phase pure cultures and incubated at 37°C for 24 hours. Pure cultures of each organism were used for comparative purposes. At different time points (0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hours), samples were taken, serially diluted in sterile phosphate-buffered saline (PBS) and plated onto Mannitol Salt agar (MSA, Difco™) and Pseudomonas Isolation agar (PIA, Difco™) to discriminate the two bacterial species. The agar plates were incubated for 24 hours at 37°C and colony forming units (CFU) were enumerated. Each experiment was repeated three times independently. The competition index (CI) for mixed culture was calculated as *P. aeruginosa*-to-*S. aureus* ratio within the output sample, divided by the corresponding ratio for the input (inoculum at time t = 0), as described by Macho and colleagues [49]. To allow an easier comparison between the variations observed in single versus mixed cultures a CI-like index, the Relative Increase Ratio (RIR) was calculated as *P. aeruginosa*-to-*S. aureus* ratio within the output sample, divided by the corresponding ratio in the inoculum, using growth results from pure cultures [49]. As the RIR is calculated on the results obtained from single growth curves, only a CI that differs statistically from the RIR of the same time-point can be considered a result of a significant competition between the species [49].

Biofilm production

Biofilm production in static conditions was visualized by crystal violet (CV) staining as previously described [34]. The following *S. aureus* and *P. aeruginosa* strains were tested: Newman, PA14, AA2 and AA43. Strains were grown overnight in Nutrient Broth (NB, Difco™) and subcultured in fresh medium for 2.5 hours to reach the mid-exponential phase of growth. Bacteria were centrifuged, pellet was washed with PBS, resuspended in fresh medium and the OD_{600} was measured to adjust the concentration of bacteria [34]. Experiments were performed in triplicate and repeated three times independently. The data were then averaged and the standard deviation was calculated.

To correlate the growth in the planktonic fraction with biofilm formation, the planktonic cell fractions, which were transferred to new microtiter plates, were quantified by plating serial dilutions on MSA and PIA agar plates. To enumerate the sessile cells of *S. aureus* and *P. aeruginosa*, the wells were rinsed three times with 200 µl of PBS to remove non-adherent and weakly adherent bacteria. Then, the biofilm was removed by scraping the surface of each well with 1 ml PBS and the recovered cells were suspended by vortexing for 30 sec. The number of sessile cells was determined by plating serial dilutions on MSA and PIA agar plates. To ensure the complete detachment of the bacteria, CV (1%) assay was performed on each of the wells scraped, and absorbance determined at 595 nm.

Mouse model of acute lung single and co-infection

Experiment of acute infection with *S. aureus* and *P. aeruginosa* strains were performed using C57Bl/6NCrlBR male mice (20 22 gr), purchased by Charles River, with minor modification to previous published protocols [20,30]. For the co-infections, *P. aeruginosa* referent strain PA14 and clinical isolates AA2 and AA43, and *S. aureus* referent strain Newman, grown at middle exponential phase, were recovered by centrifugation and resuspended in PBS

to the desired sub-lethal dose for infection of 1×10^6 CFU both for *P. aeruginosa* and *S. aureus* and mixed together at a ratio of 1:1.

C57Bl/6NCrlBR mice were anesthetized by an intraperitoneal injection of a solution of 2.5% Avertin (2,2,2-tribromethanol, 97%; Sigma Aldrich) in 0.9% NaCl and administered in a volume of 0.015 mlg^{-1} body weight. Trachea was directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula (Becton, Dickinson, Italy) attached to a 1 ml syringe. Co-infection was established with a 60 µl inoculum implanted via the cannula into the lung, with both lobes inoculated. Mice were also infected with 1×10^6 CFU of planktonic *P. aeruginosa* or *S. aureus* for comparative purposes.

After 18 hours from infection, mice were euthanized and murine lungs were aseptically excised, homogenized and plated onto MSA and PIA plates for differential CFU counting. The competition index (CI) was calculated as the ratio of *P. aeruginosa* to *S. aureus* bacteria recovered from the murine lungs after 18 hours from infection adjusted by the input ratio that was inoculated in each animal (*in vivo* CI). A CI value equal to 1 indicates equal competition of the two species; a CI value significantly <1 indicates a competitive advantage of *S. aureus* that outcompetes *P. aeruginosa*; a CI value significantly >1 indicates a competitive advantage of *P. aeruginosa* that outcompetes *S. aureus*.

Statistical analysis

In vitro agar growth inhibition data were analyzed by means of a LME (Linear Mixed effect model) separated for Newman and SH1000. Response variable was inhibition and covariates were groups (early versus late) and a random effect on patient to account for lineages heterogeneity. To analyze batch co-culture data reported in Figure 1A, 1C, 1E the CFU/ml values were transformed using a log10 function. Data retrieved from single and co-culture experiments showed a similar starting point (estimated by intercept parameter A) and different behavior in some settings over time , leading to different plateau values (estimated by parameter B) at the end of the follow-up period. This suggested to use a nonlinear mixed-effect model (ref), (with the non-linearity described by a four-parameters logistic regression function) to estimate the log10(CFU/ml) trend, modelled as it follows: $A+B/\{1+\exp[(C-x)/\exp(D)]\}$.

This kind of model is widely used for growth curve modeling. Since the parameter A represents horizontal asymptote relative to the starting point, we assign a random effect (representing the heterogeneity among experiments) on this parameter to include heterogeneity among experiments. Parameter B represents the horizontal asymptote relative to the final plateau; we studied the possible influence of single/co-culture (described by its indicator variable), in order to test the hypothesis of different plateau at the end of the follow up. This represents the main effect of interest and its significance was tested comparing likelihood with and without it. Parameter C is the inflection point and has been estimated using a maximum likelihood principle. Parameter D is strictly connected to the so called scale parameter and represents the growth rate of the logistic function. A fixed effect common for single single and co-culture was estimated. RIR and CI indexes were analyzed using Student's t-test and the null hypothesis: mean CI was not significantly different from mean RIR [49]. *In vitro* biofilm data (OD values) were analyzed using Two-tailed Student's t-test [34]. CFU biofilm data were analyzed by means of Mann Whitney test, a non-parametric procedure to evaluate a null hypothesis that two populations are the same against an alternative that one population tends to have larger values than the other. Competition index (CI) of *in vivo* experiments was calculated adapting the methods previously published [20]. To assess bacterial competition Wilcoxon signed rank test of the null hypothesis that the distribution of CI is symmetric about 1 was used. Significance was set at the usual level 0.05. All statistical analyses were performed using R 2.15.2 (http://www.R-project.org/).

Supporting Information

Table S1 ***In vitro*** **growth inhibition of** ***P. aeruginosa.*** (DOCX)

Figure S1 Single and dual species batch growth curves and competition index values. S. aureus strain (SH1000) and P. aeruginosa strains (PA14 and two clinical early and late isolates from a CF patient AA2 and AA43) were grown for 24 hours in BHI in single culture and in co-culture after inoculation at equal ratio from mid-exponential phase pure cultures. Growth rate was monitored by colony count after plating on selective media for both species. Results are represented as the mean of values obtained from three independent experiments. The error bars indicate the standard deviations. A nonlinear mixed-effect model was fitted, using a four-parameters logistic regression function. Panel A: growth curves of SH1000 in pure culture and in co-culture with PA14; Panel B: Competition index (CI) and Relative Increase Ratio (RIR) calculated from single and dual cultures of SH1000 and PA14; Panel C: growth curves of SH1000 in pure culture and in co-culture with AA2; Panel D: CI and RIR calculated from single and dual cultures of SH1000 and AA2; Panel E: growth curves of SH1000 in pure culture and in co-culture with AA43; Panel F: CI and RIR calculated from single and dual cultures of SH1000 and AA43. Each value represents the mean of CI and RIR values from three independent experiments and the bars indicate standard deviation. Statistically significant differences in Student's t test and in nonlinear mixed-effect model are indicated by symbols when present: *: $p<0.05$; ***: $p<0.001$. (TIF)

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Author Contributions

Conceived and designed the experiments: RB IB CC AB DMC. Performed the experiments: RB FT MDS IB CC. Analyzed the data: RB FT IB CC DP CDS. Contributed reagents/materials/analysis tools: CDS DMC AB. Wrote the paper: RB CC IB DP CDS AB DMC.

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Submitted paper

Host genetic background influences the response to the opportunistic *Pseudomonas aeruginosa* infection altering cell-mediated immunity and bacterial replication.

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ABSTRACT

Pseudomonas aeruginosa is a common cause of healthcare-associated infections including pneumonia, bloodstream, urinary tract, and surgical site infections. The clinical outcome of *P. aeruginosa* infections may be extremely variable among individuals at risk and patients affected by cystic fibrosis. However, risk factors for *P. aeruginosa* infection remain largely unknown. To identify and track the host factors influencing *P. aeruginosa* lung infections, inbred immunocompetent mouse strains were screened in a pneumonia model system. A/J, BALB/cJ, BALB/cAnNCrl, BALB/cByJ, C3H/HeOuJ, C57BL/6J, C57BL/6NCrl, DBA/2J, and 129S2/SvPasCRL mice were infected with *P. aeruginosa* clinical strain and monitored for body weight and mortality up to seven days. The most deviant survival phenotypes were observed for A/J, 129S2/SvPasCRL and DBA/2J showing high susceptibility and BALB/cAnNCrl and C3H/HeOuJ showing more resistance to *P. aeruginosa* infection. Next, one of the most susceptible and resistant mouse strains were characterized for their deviant clinical and immunological phenotype by scoring bacterial count, cell-mediated immunity, cytokines and chemokines profile and lung pathology in an early time course. Susceptible A/J mice showed significantly higher bacterial burden, higher cytokines and chemokines levels but lower leukocyte recruitment, particularly neutrophils, when compared to C3H/HeOuJ resistant mice. Pathologic scores showed lower inflammatory severity, reduced intraluminal and interstitial inflammation extent, bronchial and parenchymal involvement and diminished alveolar damage in the lungs of A/J when compared to C3H/HeOuJ. Our findings indicate that during an early phase of infection a prompt inflammatory response in the airways set the conditions for a non-permissive environment to *P. aeruginosa* replication and lock the spread to other organs. Host gene(s) may have a role in the reduction of cell-mediated immunity playing a critical role in the control of *P. aeruginosa* infection**.** These results now provide a basis for mapping genomic regions underlying host susceptibility to *P. aeruginosa* infection.

INTRODUCTION

P. aeruginosa is one of the major and dreaded source of infections responsible for causing millions of cases each year in the community and 10-15% of all healthcare associated infections, with more than 300,000 cases annually in the EU, North US and Japan. Patients at risk of acquiring *P. aeruginosa* are particularly those hospitalized in intensive care units (ICU) who may develop ventilator-associated pneumonia (VAP) and sepsis [137]. In general, patients with a compromised immune system, due to immunosuppressive therapies or underlying diseases such as cancer, AIDS or the hereditary disease cystic fibrosis (CF), are at risk to develop *P. aeruginosa* infection.

The clinical outcome of *P. aeruginosa* infections may be extremely variable among individuals at risk and CF patients. In particular, heterogeneity in the severity of chronic bronchopulmonary *P. aeruginosa* infection is well documented in CF, while in other patients remains to be established [138]. According to clinical studies, the progression and severity of pulmonary disease in CF do not appear to correlate with the type of *Cystic Fibrosis Transmembrane Regulator* (*CFTR)* variant and rather seem to be largely dependent on secondary factors [139]. Much influence on disease outcome has been attributed more to different *P. aeruginosa* phenotypes rather than to host genetic background. Consistent with its larger genome size and environmental adaptability, *P. aeruginosa* contains the highest proportion of regulatory genes observed for a bacterial genome which lead to large and complex phenotypic versatility. Thus, early studies from different groups including ours [140-143] highlighted the responsibility of particular *P. aeruginosa* phenotypes for differential disease manifestations and pathogenesis. For instance, the shift from the opportunistic toward a life-long persistent phenotype has a major impact in dampening the innate immune recognition and deteriorating the lung function [144]. These studies somewhat neglected the potential importance of host factors. More recently, special interest has shifted toward understanding host genetic variation that alters the outcome of *P. aeruginosa* infection [145]. Identifying and tracking risk factors for *P. aeruginosa* infection remains one of the major research challenge.

From studies of genetic predisposition in other infectious disease it has become clear that the host response is not only influenced by single genes but by combinations of genes and their variants [146, 147]. Thus, complex (multi-gene) genetic effects need to be analyzed to understand the full repertoire of host responses to pathogens. Several candidate gene association studies have been carried out in humans. However, although studies in humans are essential, they are limited because of the size of cohorts, strong but often unknown environmental influences, poor diagnosis, and lack of repeatability [145]. Therefore, animal models are absolutely essential to complement human studies.

To meet the current challenge of deepening genetic susceptibility to infection and dissection of genetic traits analysis, well defined mouse genetic reference populations (GRP) have been a powerful force. Mouse GRP are available as inbred laboratory and wild-derived mouse strains, recombinant inbred strains, interspecific recombinant inbred strains, chromosome substitution strains, and consomic strains [148]. More than 200 commercially available, phylogenetically diverse inbred mouse strains that contain enough genetic diversity to identify major differences in response to a specific infection are available [149]. These resources have

been extensively used to identify cellular and molecular factors that may contribute to different disease pathogenesis and to analyze the effect of multiple contributing gene loci influencing disease phenotype with different pathogens [146, 150]. Successful stories included gene mapping for a large number of pathogens like bacteria (e.g. *Salmonella enterica serovar Typhimurium, Mycobacterium bovis, Bacillus anthracis, Staphilococcus aureus* and *Legionella pneumophila*), parasites (e.g. *Plasmodium chabaudi, Candida albicans,* and *Leishmania donovani*) and viruses (e.g *Cytomegalovirus, Vescicular stomatitis virus* and *Orthomyxovirus)* [149, 151]. Surprisingly, to our knowledge this approach has not been used to map for genetic determinant(s) of *P. aeruginosa* infection.

As a first step toward the analysis of genetic traits influencing resistance and susceptibility to *P. aeruginosa* infection and the characterization of pathogenetic mechanims, we screened nine inbred mouse strains of differing ancestry and chosen for the known differences in their ability to overcome infections with various pathogens. Using characterized mouse model of acute infection with *P. aeruginosa* clinical strains and previous experience in this model system [152, 153], we identified mouse strains presenting deviant clinical and immunological phenotypes amenable for biological and genetic analyses.

RESULTS

Survival and body weight of *P. aeruginosa*-infected inbred mice is strongly dependent upon genetic background.

Nine different inbred mouse strains, namely A/J, BALB/cJ, BALB/cAnNCrl, BALB/cByJ, C3H/HeOuJ, C57BL/6J, C57BL/6NCrl, DBA/2J, and 129S2/SvPasCRL were infected with $5x10^6$ CFU of planktonic *P. aeruginosa* clinical isolate AA2 via intra-tracheal injection, and monitored for change in body weight and mortality over a period of seven days. As shown in **Fig. 1,** a wide-range of survival and weight loss were observed among different inbred mice. The most deviant survival phenotypes were observed for A/J, 129S2/SvPasCRL and DBA/2J showing high susceptibility and BALB/cAnNCrl and C3H/HeOuJ showing more resistance to *P. aeruginosa* infection. BALB/cJ, BALB/cByJ, C57BL/6J, C57BL/6NCrl showed intermediate phenotype. In more detail, susceptible A/J, 129S2/SvPasCRL and DBA/2J died within the first two days of infection, showed a mean survival time of around one day and a rapid and fatal decrease of body weight (**Fig. 1A, B** and **C**). Within the susceptible mice, A/J were significantly different compared to DBA/2J and 129S2/SvPasCRL showing a faster decrease of body weight at day one (**Table S1**) and a kinetic of death significantly more rapid (**Fig. 1A**). Resistant mice BALB/cAnNCrl and C3H/HeOuJ showed a significant lower susceptibility to *P. aeruginosa* infection compared with A/J, DBA/2J and 129S2/SvPasCRL with cases of survival, a mean survival time of at least three days and a progressive weight recovery of the survivors at day seven (**Fig. 1A, B** and **C**). Within the resistant mice, BALB/cAnNCrl and C3H/HeOuJ were not significantly different for the body weight, kinetic of death and survival time (**Table S1** and **Fig. 1**). The above described differences in resistance and susceptibility of most deviant inbred mice were confirmed by infecting with a lower *P. aeruginosa* infection dose of $5x10^5$ CFU (**Table S2 and Figure S1**).

Impaired cell-mediated immunity leads to faster replication of *P. aeruginosa* in A/J mice when compared to C3H/HeOuJ.

Next, one of the most susceptible and resistant mouse strains were characterized for their deviant clinical and immunological phenotypes after *P. aeruginosa* AA2–induced acute pneumonia. The *P. aeruginosa* load and immune response of infected mice in terms of leukocyte recruitment, myeloperoxidase activity, and local cytokine production in the airways were investigated in A/J and C3H/HeOuJ mice during an early time course (6, 12 and 18 hours post-infection). Starting from a challenge of $5x10^6$ CFU, significant increase of total bacterial load up to 2 $\log_{10}$ CFU ($4.1x10^8$) at 18 hours in the lung of susceptible A/J mice was observed indicating an uncontrolled replication of bacterial cells (**Fig. 2A**). Conversely at the same time points, the bacterial load in the lungs of resistant C3H/HeOuJ mice was unchanged in respect to the initial inoculum (6.9×10^6) suggesting that resistant mice are able to keep in check the infection. A/J susceptible and C3H/HeOuJ resistant mice were significantly different in their bacterial load at all time points. Similar differences were also observed in different airways compartments as assessed by the Broncho Alveolar Lavage Fluid (BALF) (**Fig. 2B**) and lung homogenate analysis (**Fig. 2C**).

Leukocyte recruitment in the BALF of susceptible A/J mice early after *P. aeruginosa* infection was significantly lower than C3H/HeOuJ resistant mice (**Fig. 2D**). Furthermore, while the low leukocyte numbers remained stable in A/J during 18 hours, their recruitment in C3H/HeOuJ increased and reached the pick at 12h. In particular, a significant increase in neutrophil levels for C3H/HeOuJ, compared to A/J was observed during 18h (**Fig. 3A**). The higher level of myeloperoxidase (MPO) activity in the BALF of C3H/HeOuJ compared to A/J supported these data (**Fig. S2**). Macrophages were also significantly higher in the lung of C3H/HeOuJ mice compared to A/J mice at 12 hours post infection, but no striking differences were present at 6 and 18 hours post infection (**Fig. 3B**). Lymphocytes and epithelial cells showed a trend being higher in C3H/HeOuJ mice but did not reach significance (**Fig. 3C and D**).

When CFUs and neutrophils recovered in the BAL were plotted together, a distinct trend was observed in A/J and C3H/HeOuJ mice during 18 hours of infection. **Fig 4** showed a lower number of neutrophils recruited and a higher number of *P. aeruginosa* CFUs in A/J mice in comparison to C3H/HeOuJ mice. In particular, looking at the ratio of *P. aeruginosa* CFUs and neutrophils, significant differences were found (**Fig S3)**. During the whole time-course, the ratio between CFUs and neutrophils was significantly lower for C3H/HeOuJ mice when compared to A/J mice, indicating a higher capacity of C3H/HeOuJ mice to control *P. aeruginosa* infection.

A/J mice showed higher cytokines and chemokines profile in the airways when compared to C3H/HeOuJ mice during acute *P. aeruginosa* pneumonia.

To better characterize the airway inflammatory response of the two deviant inbred mouse strains, we measured the concentration of a large panel of twenty-three cytokines and chemokines in murine lung homogenates. As shown in **Table 1**, the overall levels of pro-inflammatory cytokines were significantly higher in the lung of A/J mice respect to C3H/HeOuJ mice and reach significance for IL-17, G-CSF, GM-CSF, IFN-γ, IL-6, IL-1α, KC, MCP1 and MP1 in at least one time point. Other cytokines, TNF-α and IL-5, were higher in A/J compared to C3H/HeOuJ mice but did not reach significance. Thus, A/J mice showed an excessive release of pro-inflammatory cytokines that does not correlate with cellular response when compared to C3H/HeOuJ mice.

Pathological differences in A/J and C3H/HeOuJ mice during *P. aeruginosa* acute pneumonia.

The histopathologic analysis of *P. aeruginosa* AA2–induced acute pneumonia revealed striking differences between A/J and C3H/HeOuJ mice (**Fig. 5** and **Fig. S4**). During an early time course a fast and consistent recruitment of inflammatory cells in C3H/HeOuJ mice compared to a delayed and lower recruitment in A/J was observed (**Fig. 5 A-C, E-G**).

More in detail, during early phases of infection pathologic scores showed that the high inflammatory response of C3H/HeOuJ resistant mice was characterized by strong polymorphonuclear leukocytes (PMNs) recruitment in BALF and in the interstitial areas of the lungs followed by a progressive increase in macrophages involvement and a moderate expansion of bronchus associated lymphoid tissue (BALT)-like

structure (**Fig. 5 A-C, E-G** and **Fig. S4**). These cell-mediated response may have a key role in mounting an effective response to control bacterial clearance. Indeed, immunofluorescence staining showed low number of *P. aeruginosa* cells in the lungs of C3H/HeOuJ mice (**Fig. 5H**).

The susceptible A/J strain responded in a delayed manner characterized by a lower cell recruitment associated to low damage in the lung, and absence of a relevant interstitial/BALF response (**Fig. 5 A-C, E-G** and **Fig. S4**). The BALT-like structure observed in C3H/HeOuJ mice was substantially never observed in A/J susceptible strain. Indeed, immunofluorescence staining showed high numbers of *P. aeruginosa* cells localized both within the bronchial lumen and in the alveolar space indicating inadequate response of A/J mice to control bacterial replication in the lung (**Fig. 5D**).

DISCUSSION

An increasing number of papers reports that the host genetic background significantly modulates the outcome of infectious diseases highlighting that in most cases multiple complex genetic interactions may have a key role in controlling infection [146]. These findings reveal that pathogens and their associated phenotypes are not the only determinants of the corresponding infectious diseases. In the case of *P. aeruginosa* opportunistic infection, much influence on disease outcome has been mainly attributed to different bacterial phenotypes rather than host genetic background. Here, we selected nine inbred murine strains characterized by a wealth of genetic and phenotypic diversity and representing a discrete part of the numerous hallmarks of the human population [149]. We show that different inbred murine strains are highly variable in their response to acute airway infection. During a time course analysis, a wide-range response to *P. aeruginosa* infection has been observed both in the survival rate and body weight change of different inbred murine strains. Most notably, deviant clinical phenotypes were observed being the A/J, 129S2/SvPasCRL and DBA/2J as the most susceptible while BALB/cAnNCrl and C3H/HeOuJ the most resistant murine strains. Other murine strains (BALB/cJ, BALB/cByJ, C57BL/6J and C57BL/6NCrl) showed intermediate phenotype. Furthermore, we demonstrate no significant behavioral differences among distinct sub-strains of C57BL/6 from Jackson (C57BL/6J) or Charles River (C57BL/6 NCrl). As reported in other studies, recent observations suggest that different variants of C57BL/6 mice are similar when monitored for acute mortality but have a different susceptibility to *P. aeruginosa* infection in terms of chronic persistence [141]. When distinct sub-strains of BALB/c from Jackson (BALB/cJ and BALB/cByJ) or Charles River (BALB/cAnNCrl) were compared, we did not find any obvious differences. In this context, it is interesting to note that the widely used CB57BL/6 mice were not the most susceptible to *P. aeruginosa* infection compared to other inbred strains. C57BL/6 is also the background of *Cftr*-ko mice and it is known that these mice do not mimic human CF lung disease as in humans [152]. Our results open the question whether other mouse genetic background may better recapitulate the pulmonary abnormalities of CF patients during *P. aeruginosa* infection.

Some of the inbred mouse strains used in this work were tested previously by others employing different model systems of *P. aeruginosa* infection. So far, three papers directly compared more than four inbred mouse strains [127, 133, 154]. Intraperitoneal *P. aeruginosa* infection of 16 different strains of inbred mice showed enhanced resistance for C3H mice and susceptibility for A.BY [127]. Most strains, including DBA/2J, C57BL/6J and BALB/c, used also in our work, showed no significant differences. In another work, 11 inbred mouse strains were compared using aerosol model of *P. aeruginosa* infection [133]. DBA/2J were the most susceptible while A/J, C3H/HeN, SWR/J and B10.D2/nSnJ were the most resistant when monitored for mortality. Using a chronic model of *P. aeruginosa* infection and evaluating mortality and bacterial load, a more restricted number of four inbred mouse strains were compared. BALB/c mice were found to be resistant, and DBA/2J mice were identified as the most susceptible strain while C57BL/6NHsd and A/JCr mice were found to be relatively susceptible [154]. Additional papers characterized inflammatory response

directly comparing one resistant and one susceptible mice considering as resistant and susceptible BALB/c and C57BL/6 [155, 156], BALB/c and DBA/2 [157], C3H/HeN and BALB/c respectively [158, 159]. Taken together our results and those of previous reports, it seems that a comprehensive classification of mouse inbred strains into categories according to their resistance or susceptibility is difficult to achieve. As demonstrated for other pathogens, the differences in the disease outcome may be affected by the site of infection [160, 161]. In the above mentioned works, different routes of administration have been used for *P. aeruginosa* infection including intraperitoneal [127], aerosol [133] or intratracheal (this work and [154]). Other factors have also been shown to influence the disease phenotype and, by interference, susceptibility or resistance. The size of the inoculum and the strain of the pathogen used seem to be particularly important. Different *P. aeruginosa* bacterial strains have been used, including laboratory PAO1 [133] or clinical strains PA-103 [127], PA-508 [154] and AA2 in this work. These results suggest a previously unrevealed level of complexity and show that conclusions regarding disease susceptibility induced by *P. aeruginosa* infection may be highly dependent on the experimental model that is used.

Mouse inbred strains are the starting point from which to explore causal phenotype-genotype relationships, including the identification of cell-mediate immune response and gene mapping. In this work, the susceptible A/J mice and the resistant C3H/HeOuJ have been used to gain deeper insight into the cellular and molecular factors that may contribute to different disease pathogenesis. High bacterial replication and inadequate immune-response was observed for A/J mice suggesting that susceptible mice are inefficient to keep in check the bacterial infection. In other words, A/J mice do not mount a proper, early immune defence leading to a permissive environment for bacterial replication and spreading from the airways to other organs and ultimately to a fast disease progression. The lack of the inflammatory response in A/J mice may not be attributable to deficient cytokines or chemokines response, rather mice showed an excessive or uncontrolled release of pro-inflammatory cytokines, suggesting an ongoing cytokine storm. Previous studies have demonstrated that A/J mice are known to carry a loss-of-function mutation at the C5 locus, which is implicated in mobilizing inflammatory cells, in particular neutrophils, critical for host defense against infection [134]. This deficiency has been widely demonstrated to account for the susceptibility of A/J mice to several pathogens, such as *Candida albicans* [162] and *Mycobacterium tuberculosis* [163]. However, deficiency of C5 may not be completely responsible for *P. aeruginosa* susceptibility since inbred strains deficient (A/J and DBA/2) or non-deficient for C5 (129S2/SvPasCRL) are equally susceptible to *P. aeruginosa* infection.

In contrast to susceptible A/J mice, resistant C3H/HeOuJ mice mount a faster and consistent immune-response that is able to efficiently control bacterial replication. In more detail, C3H/HeOuJ mice exhibited a prompter recruitment of inflammatory cells, mainly PMN, to the site of infection than did mice of the A/J strain. A balanced level of cytokines or chemokines has been observed in C3H/HeOuJ mice. Furthermore, after a strong PMNs response of C3H/HeOuJ resistant mice, a progressive increase in macrophages involvement and BALT-like structure activation was observed. This BALT-like structure activation, instead, was substantially never observed in A/J susceptible strain. These differences clearly indicates that, in this

model of infection, the two mouse strains react differently in modulation of inflammatory response and probably in the way of the antigen presentation to the lymphocytes of BALT. These cells may play a role in mounting a cell-mediated response to determine effective control or bacterial clearance.

Other papers on inbred mice analysed the inflammatory response in susceptible and resistant strains showing controversial results about the biological significance of the early inflammatory response in modulating the course and outcome of *P. aeruginosa* infection. In agreement with our results, Morissette and co-workers reported a rapid influx of PMN which was shortly followed by an efficient clearance of bacteria in BALB/c resistant mice, while DBA/2 susceptible mice had a delay in both the inflammatory response and the initiation of bacterial clearance [154, 157]. However, other papers report an accumulation of inflammatory cells in susceptible rather than resistant mice. An exaggerated inflammatory response dominated by PMNs correlates with susceptibility to infection in C57BL/6 mice, whilst a modest inflammatory response dominated with macrophages correlated with resistance in BALB/c mice [155, 156]. Nevertheless, it should be expected that differences in the experimental model used (acute vs chronic) may affect several physiological parameters, adding complexity to the overall picture of the *P. aeruginosa*/host interaction.

Taken together, our results showed that survival to *P. aeruginosa* infection is clearly affected by genetic background. Comparative analysis of the cell-mediated immunity to *P. aeruginosa* infection in resistant and susceptible strain has been used in determining key player of a successful versus an unsuccessful response to infection. During this early phase of infection, a prompt inflammatory response in the airways provides a biological advantage in creating a non-permissive environment for *P. aeruginosa* replication and locking the spread to other organs. Thus, we speculate that host gene(s) may have a role in the reduction of cell-mediated immunity playing a critical part in the control of *P. aeruginosa* infection. With the use of recombinant inbred strategies, the survival differences between A/J and C3H/HeOuJ mice will permit future mapping of key genes involved in *P. aeruginosa* infection.

MATERIALS AND METHODS

Ethic Statement.

Animal studies were conducted according to protocols approved by San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals (Permit number: 502). Research on the bacterial isolates from the individual with CF has been approved by the responsible physician at the CF center at Hannover Medical School, Germany. Patient gave informed consent before the sample collection. Approval for storing of biological materials was obtained by the Hannover Medical School, Germany

Bacterial strain.

P. aeruginosa clinical isolate AA2 was obtained from a CF patient attending the Medizinische Hochschule of Hannover, Germany at the onset of chronic colonization and described before [141, 153]. The strain was cultured in trypticase soy broth (TSB) and plated on trypticase soy agar (TSA).

Mouse model of *P. aeruginosa* acute infection.

Nine inbred immune-competent mouse strains eight weeks old namely A/J, BALB/cJ, BALB/cByJ, C3H/HeOuJ, C57BL/6J and DBA/2J were purchased from Jackson (J) and BALB/cAnNCrl, C57BL/6NCrl, 129S2/SvPasCRL from Charles River laboratories (Crl). Mice were infected with the doses of $5x10^5$ and $5x10^6$ CFU of planktonic *P. aeruginosa* clinical isolate AA2 according with established procedures [153]. Briefly, mice were anesthetized by an intra-peritoneal injection of a solution of 2.5% Avertin (2,2,2-tribromethanol, 97%; Sigma Aldrich) administered in a volume of 0.015 mlg^{-1} body weight and the trachea directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe. A 50 µl inoculum of $5x10^5$ or $5x10^6$ CFU were implanted via the cannula into the lung, with both lobes inoculated. After infection, mortality and body weight were monitored over one week. Animals showing more than 25% of body weight loss were euthanized and documented as dead. For the time course, additional groups of mice were sacrificed by CO_2 administration at 6, 12 and 18 hours and analysed for CFU in the lung, BALF cell count and myeloperoxidase (MPO) activity as previously described [164, 165]. In another group of mice, the lungs were excised and used for histopathology.

BALF and lung collection and analysis.

BALF was extracted with a 22-gauge venous catheter by washing the lungs with RPMI-1640 (Euroclone) with protease inhibitors (Complete tablets, Roche Diagnostic and PMSF, Sigma) [166]. Total cells present in the BALF were counted, and a differential cell count was performed on cytospins stained with Diff Quick (Dade, Biomap, Italy). BALF was serially diluted and plated on TSB-agar plates. After erythrocyte lysis with ACK lysis solution (pH 7.2; NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA 0.1 mM), cells were resuspended in cetyltrimethylammonium chloride 0.5% (Sigma Aldrich) and centrifuged. The clear extracts were used to

analyse the MPO activity: samples were mixed with equal volumes of 3,3′,5,5′-tetramethyl-benzidine dihydrochloride for 2 min and the reaction stopped by adding H_2SO_4. The OD was measured at 450 nm.

Lung homogenization and cytokine analysis.

Lungs were removed and homogenized in PBS with Ca2+/Mg2+ containing protease inhibitors. Samples were serially diluted and plated on the above agar media for CFU counts. Lung homogenates were then centrifuged at 14,000 rpm for 30 minutes at 4°C, and the supernatants were stored at – 20°C for cytokine analysis.

Total lung homogenates protein content was quantified with Bradford's assay (Bio-RAD) at the final concentration of 500µg /ml. A panel of 23 murine chemokines and cytokines were measured using Bio-Plex pro™ Mouse Cytokine Standard 23-Plex, Group I according to the manufacterer's instructions.

Histologic and immunofluorescence analysis.

Lungs were removed, fixed in 10% buffered formalin for at least 24 h and embedded in paraffin. Consecutive 2-mm sections from the middle of the five lung lobes were used for histological and immunofluorescence examination in each mouse. Sections for histological analysis were stained by Haematoxylin-Eosin according to standard procedures. To grade inflammation severity and extent (e.g diffuse, intraluminal and/or interstitial), alveolar damage, bronchial involvement, and percentage of parenchyma involved histological score analysis of murine lungs was performed as described in the Supplementary Materials.

Localization of *P. aeruginosa* was performed in de-paraffinized lung sections by employing a rabbit antiserum specific for *P. aeruginosa* and Texas Red-labelled goat anti-rabbit IgG as described [141]. The slides were examined using an Axioplan fluorescence microscope (Zeiss) and images were taken with a KS 300 imaging (Kontron).

Statistical analysis.

Results are presented as median or mean ±SEM. Because of the small sample size and the non-normal distribution of variables, we used a nonparametric approach for most part of analysis. The Mann –Whitney U test was used to compare CFU, cells, MPO activity, cytokines levels and histologic measurements between A/J and C3H/HeOuJ mice. Mantel–Cox test was used to determine the significance of differences in means between pairs. Two- way ANOVA with Bonferroni's Multiple Comparison test was used to compare change in body weight. One-way ANOVA with Bonferroni's Multiple Comparison test was used to compare the mean survival time. Tests are considered statistically significant when the significance level is ≤ 0.05

TABLE

Table 1. Cytokines and chemokines levels in lung homogenates of susceptible A/J and resistant C3H/HeOuJ mice infected with *P. aeruginosa* during a time course.

	A/J			C3H/HeOuJ			A/J vs C3H/HeOuJ		
Cytokines	**6h**	**12h**	**18h**	**6h**	**12h**	**18h**	**6h**	**12h**	**18h**
IL1α	777.405	443.0235	157.775	378.485	489.875	241.995	*	ns	*
IL1β	726.39	1151.41	393.915	462.985	492.23	302.1	Ns	ns	Ns
IL2	nd	nd	nd	nd	nd	nd	-	-	-
IL3	3.485	3.465	1.785	1.505	1.835	1.385	Ns	*	Ns
IL-4	15.595	13.555	12.085	9.675	12.67	10.725	Ns	ns	Ns
IL5	8.62	7.42	5.165	4.205	4.05	5.675	Ns	ns	Ns
IL6	473.33	378.19	193.035	305.56	201.27	176.795	Ns	*	Ns
IL9	35.7	36.43	48.495	35.705	35.7	55.195	Ns	ns	Ns
IL10	39.25	49.095	32.04	20.44	30.75	23.51	*	*	Ns
IL12p40	56.4	21.655	23.855	14.59	19.8	21.21	*	ns	Ns
IL-12p70	254.15	182.17	113.995	164.805	228.87	147.245	Ns	ns	Ns
IL13	71.26	74.4	73.515	40.14	55.46	65.09	*	*	Ns
IL17	18.945	20.26	22.41	9.825	12.41	14.525	*	*	Ns
eotaxin	nd	nd	nd	nd	nd	nd	-	-	-
G CSF	1778.86	5840.23	6713.8	607.365	2067.78	3266.77	*	*	Ns
GM CSF	170.26	156.01	151.21	124.38	129.5	123.595	Ns	*	Ns
IFNγ	8.43	6.69	1.97	2.545	7.55	2.105	*	ns	Ns
KC	68190	69190	69290	5793.96	6747.85	3068.26	*	ns	Ns
MCP1	2826.25	1752.27	1109.07	757.01	1503.73	731.82	*	ns	Ns
MIP1α	963.815	711.72	577.235	270.05	487.66	365.4	*	ns	Ns
MIP1β	62.63	69.04	58.95	42.735	44.59	37.83	*	ns	Ns

RANTES	32.67	45.24	27.125	15.22	25.115	34.03	Ns	ns	Ns
TNFα	18.08	15.105	16.6	9.76	12.38	15.105	Ns	ns	Ns

Data are expressed as median of pg/500ug lung.
Statistical analysis for comparison of A/J vs C3H/HeOuJ at each time point by the non-parametric Mann-Whitney U test (*$p<0.05$) is reported.
Nd: not detectable; ns: not significant.

FIGURE

Figure 1: Survival, body weight and mean survival time after *P. aeruginosa* infection in inbred mouse strains.
A/J (n=22), BALB/cJ (n=9), BALB/cAnNCrl (n=8), BALB/cByJ (n=12), C3H/HeOuJ (n=26), C57BL/6J (n=10), C57BL/6NCrl (n=15), DBA/2J (n=12), and 129S2/SvPasCRL (n=12) mice were inoculated with $5x10^6$ CFU of *P. aeruginosa* clinical isolate AA2, and monitored for survival (**A**) and weight change for a period of seven days after infection (**C**). In addition, mean survival time was calculated based on the survival curve (**B**). The data are pooled from two to four independent experiments. Statistical significance by Mantel-Cox test for survival (**A**), One- way ANOVA with Bonferroni's Multiple comparison test (**B**) for mean survival time (*p<0.05, **p<0.01, ***p<0.001). Statistical significance by Two-way ANOVA with Bonferroni' s Multiple comparison test for body weight is reported in **Table S1**.

Figure 2: *P. aeruginosa* load and leukocytes recruitment in BALF and lung of A/J and C3H/HeOuJ mice.
A/J (n=12, for each time) and C3H/HeOuJ (n=12, for each time) mice were challenged with $5x10^6$ CFU of AA2 clinical stain and analysed during a time course post-infection. Bacterial loads in the total lung (**A**) including BAL (**B**) and lung homogenate (**C**) were counted at 6, 12 and 18 hours in surviving mice. Dots represent CFU per lung in individual mice and horizontal lines represent median values reported in log scale. Total leukocytes were analyzed in BALF of *P. aeruginosa* infected mice (**D**). Bars represent median values. Blue is referred to A/J and green to C3H/HeOuJ. The data are pooled from two independent experiments. Statistical significance by Mann-Whitney U test is indicated: **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3: Lung inflammatory response in susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice.
The number of neutrophils (**A**), macrophages (**B**), lymphocytes (**C**) and epithelial cells (**D**) recruited in the airways were determined in the BALF from A/J (n=12) (blue bar) and C3H/HeOuJ (n=12) (green bar) mice after 6, 12 and 18 hours of *P. aeruginosa* infection with $5x10^6$ CFU of AA2 clinical isolate. The data are pooled from two independent experiments. Statistical significance by Mann-Whitney U test is indicated: *p<0.05, **p<0.01, ***p<0.001.

Figure 4: Correlation of CFU/neutrophils in the BAL of susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice.
CFUs and neutrophils recovered in the BAL were plotted for the two murine strains during 18 hours of *P. aeruginosa* infection. Blue dots represent A/J mice (n= 12, for 6 and 12 hours, n= 9 for 18 hours) and green dots C3H/HeOuJ mice (n= 12 for each time). The data are pooled from two independent experiment.

Figure 5: Histopathology in susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice.
The lungs of A/J (A-D) and C3H/HeOuJ (E-H) were stained with H&E (A-C) and in immunofluorescence with specific antibody against *P. aeruginosa* (red) (D, H). Counterstaining was performed with 49,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (blue). After time course analysis, extensive infection and inflammation were visible in murine lungs with major differences between A/J and C3H/HeOuJ. Arrows indicate BALT-like structure. Bars, 200 µm for H&E images, 100µm for immunofluorescence images. Severity of lesions and lung involvement was scored as reported in **Fig S4**.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MDS LS NIL CC AB. Performed the experiments: MDS LS NIL GR IDF. Analyzed the data MDS LS NIL GR IDF CC. Wrote the paper: MDS LS AB.

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Figure 1
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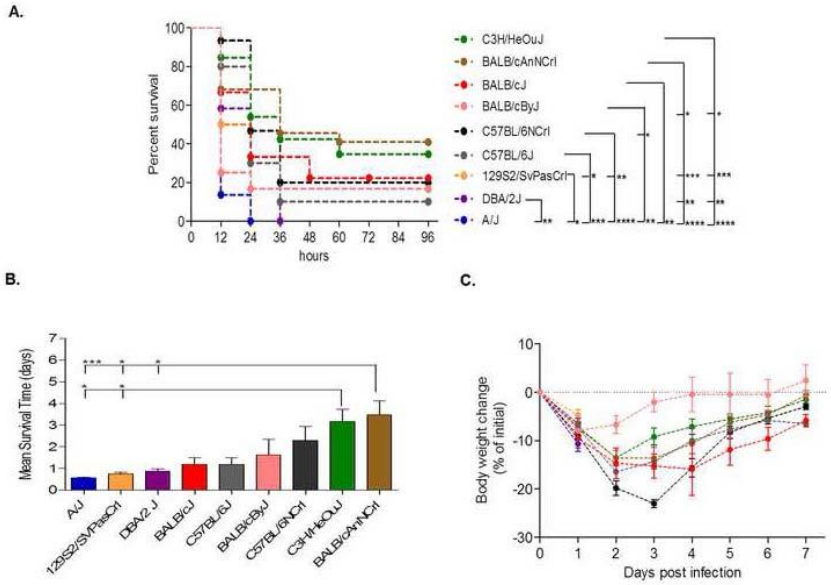

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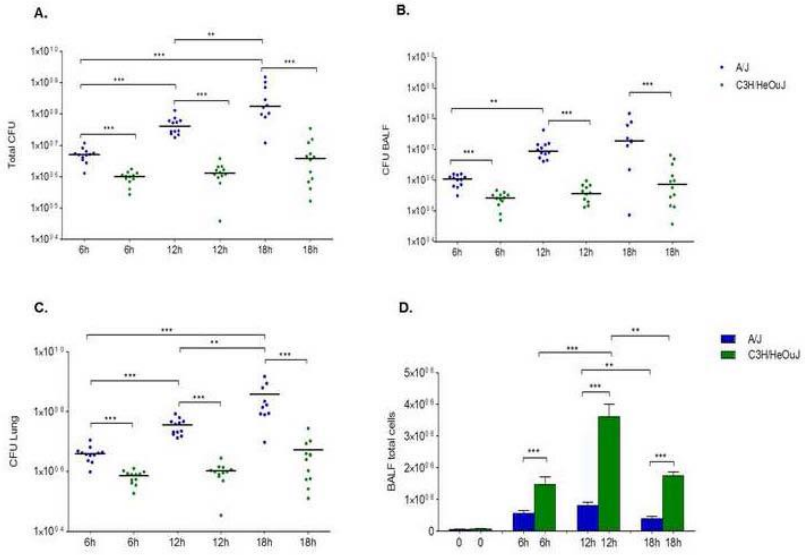

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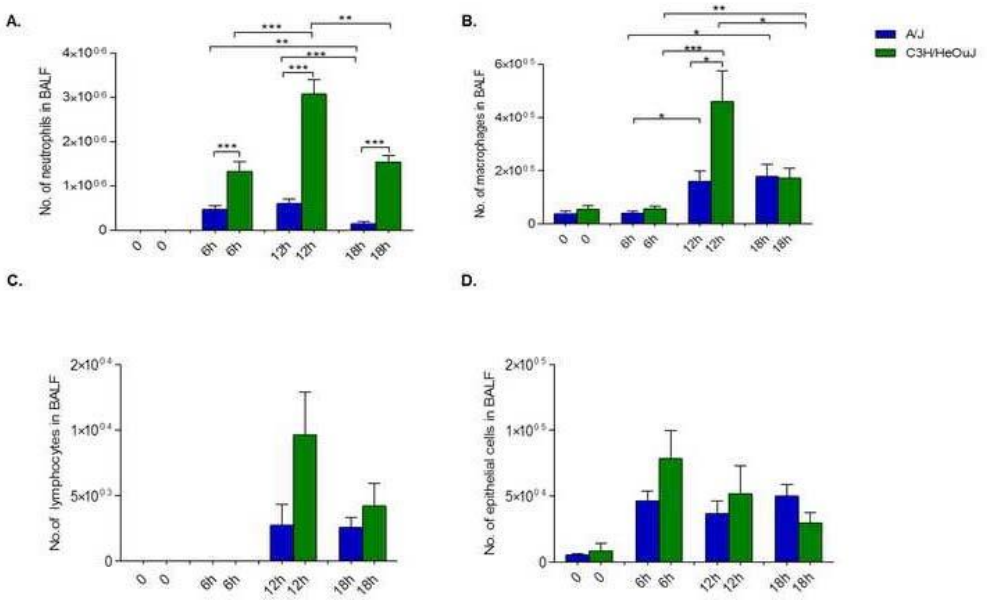

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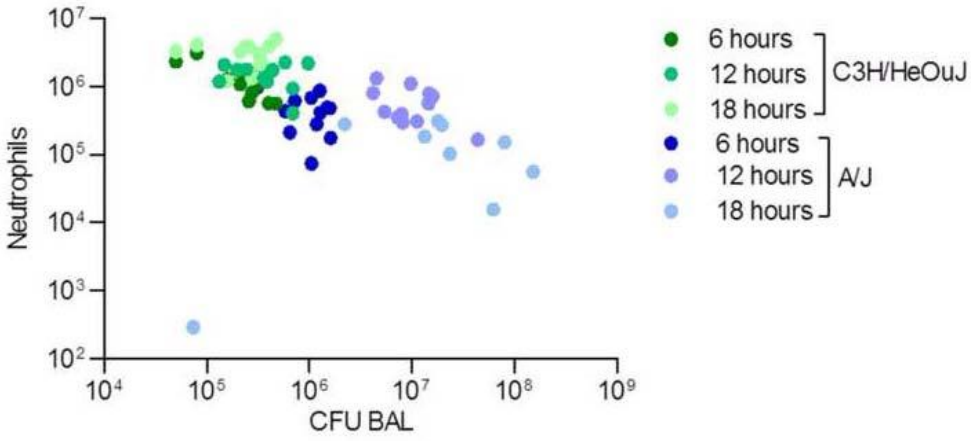

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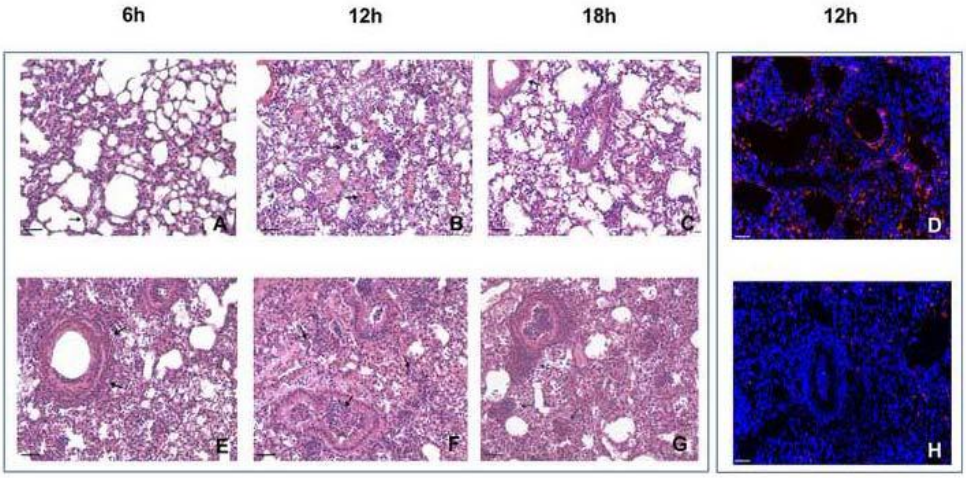


Supplementary materials

MATERIALS AND METHODS

Histologic analysis.

To grade inflammation severity and extent (e.g diffuse, intraluminal and/or interstitial), alveolar damage, bronchial involvement, and percentage of parenchyma involved histological score analysis of murine lungs was performed. Histological examination primarily included the assessment of inflammation by scoring the number of inflammatory cells (mononuclear cells, such as macrophages, lymphocytes, and plasma cells, and neutrophils) at a magnification of 400. The number of inflammatory cells was evaluated by using a visual analogue scale modified for murine pulmonary specimens, as described previously {Cersini, 2003 #803}, and results are reported as the mean for the entire specimen. When considerable variation of intensity of infiltration was evident in the same specimen, the mean for several areas was determined and the specimen was scored accordingly. Neutrophils and mononuclear cells were classified as absent (score of 0) when there were no or fewer than 19 cells per high-power field (HPF) (at a magnification of 400), mild (score of 1) for 20 to 49 cells per HPF, moderate (score of 2) for 50 to 99 cells per HPF, marked or severe (score of 3) for 100 to 200 cells or more per HPF. Percent of parenchyma involved was scored as 0 when no area of the two lungs was interested. Considering 100 the entire area of the two lung sections performed in the sagittal direction, the involvement of the parenchyma was scored as 1 when approximately the 25% of the total area was occupied by inflammatory exudate; was scored as 2 when the involved area was comprised between 26 to 50%, 3 if comprised between 51 to 75%, and 4 if between 76 to 100%.

Histological criteria for normal pulmonary characteristics included detection of no or only a few mononuclear cells per HPF and no or only a few scattered neutrophils in bronchioli and alveoli without tissue changes (no interstitial thickening or bronchiolar-associated lymphoid tissue [BALT] activation and airways free from exudate).

FIGURE

Figure S1: Survival, body weight and mean survival time after *P. aeruginosa* infection in inbred mouse strains.

A/J (n=19), BALB/cAnNCrl (n=8), C3H/HeOuJ (n=17), C57BL/6NCrl (n=15) and 129S2/SvPasCRL (n=8) mice were inoculated with $5x10^5$ CFU of *P. aeruginosa* clinical isolate AA2, and monitored for survival (**A**) and weight change for a period of seven days after infection (**C**). In addition, mean survival time was calculated based on the survival curve (**B**). The data are pooled from two to three independent experiments. Statistical significance by Mantel-Cox test for survival (**A**), One- way ANOVA with Bonferroni's Multiple comparison test (**B**) for mean survival time (*p<0.05, **p<0.01, ***p<0.001). Statistical significance by Two-way ANOVA with Bonferroni's Multiple comparison test for body weight is reported in **Table S1B**.

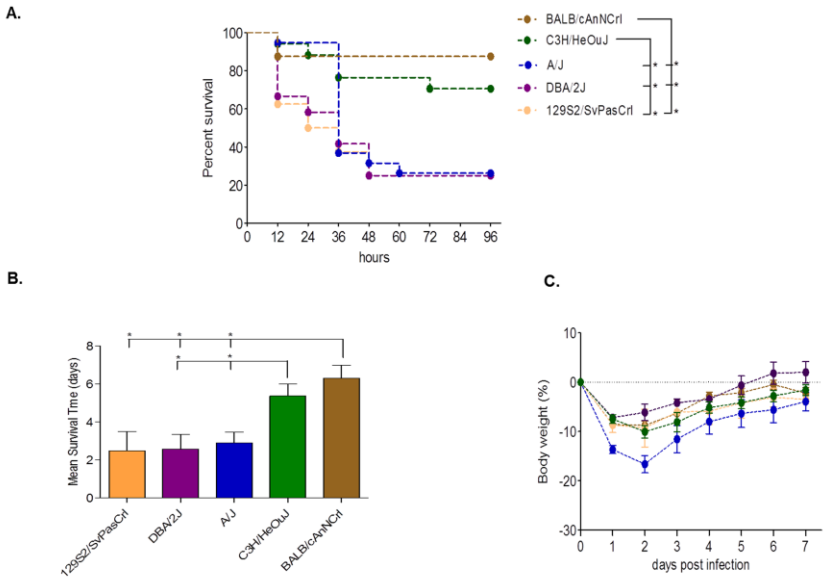
Figure S2: Myeloperoxidase activity in susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice. MPO activity was measured in the BALF of A/J (n= 12) and C3H/HeOuJ (n= 12) mice after 6, 12 and 18 hours of infection with 5 $x10^6$ CFU of AA2 clinical isolate. The data are pooled from two independent experiments. Statistical significance by Mann- Whitney U test is indicated: ***p<0.001

Figure S3: Ratio CFU /neutrophils in the BALF of susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice. A/J (n=12 for 6 and 12 hours, n=9 for 18 hours) and C3H/HeOuJ (n= 12 for each time) showed a significant different ratio of CFU /neutrophils in the BALF during the whole time-course of infection. Dots represent the CFU /neutrophils ratio in individual mice and horizontal lines represent median values. The data are pooled from two independent experiments. Statistical significance by Mann-Whitney U test is indicated: *p<0.05, ***p<0.001, ****p<0.0001.

Figure S4: Histopathology in susceptible A/J and resistant C3H/HeOuJ *P. aeruginosa*-infected mice.

Histological score analysis of A/J and C3H/HeOuJ lungs was performed at 6, 12 and 18 hours evaluating the degree of inflammation severity (A), inflammation extent–diffuse intraluminal (B), inflammation extent-interstitial (C), alveolar damage (D), bronchial involvement (E), percentage of parenchyma involvement (F). Statistical significance by Mann-Whitney U test is indicated: * p<0.05, ** p<0.01

Figure S1



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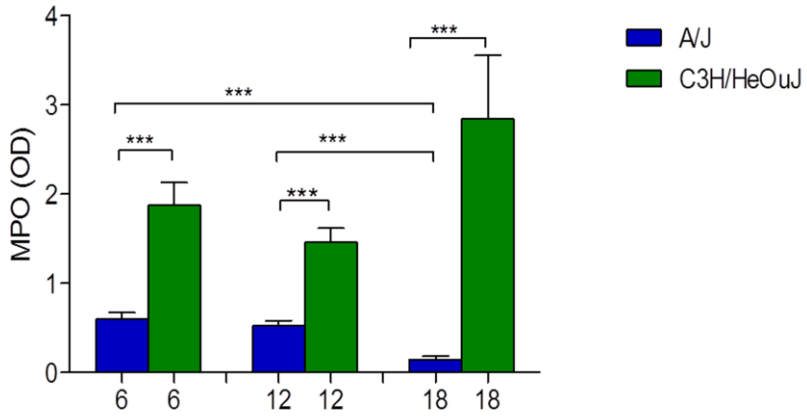

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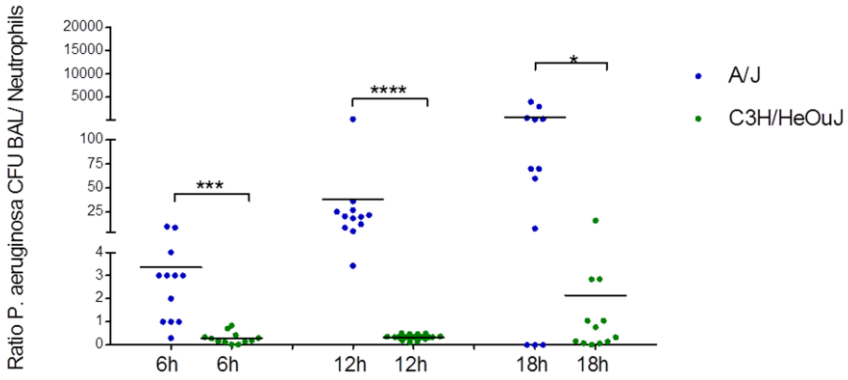

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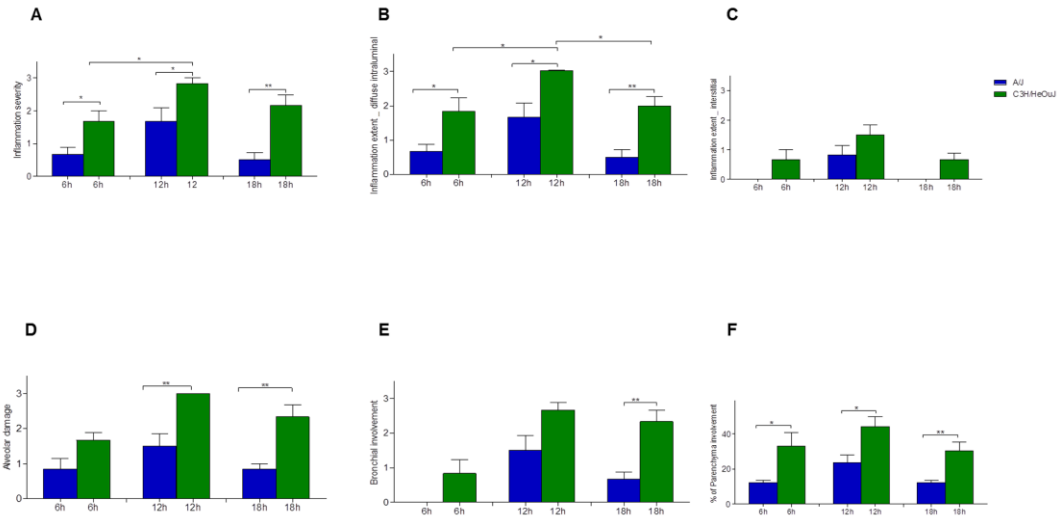

TABLES

Table S1. Statistical comparison of change in body weight between inbred mice infected with $5x10^6$ *P. aeruginosa*.

Strain	129S2/SvPasCrl	DBA/2J	C57BL/6J	BALB/cBJ	C57BL/6NCrl	C3H/HeOuJ	BALB/cAnNCrl
A/J	***	*	ns	ns	ns	ns	ns
C57BL/6NCrl	ns	ns	ns	***	-	ns	ns
C3H/HeOuJ	ns	ns	ns	ns	***	ns	ns
C57BL/6NCrl	ns	ns	*	****	-	ns	*
BALB/CAnNCrl	ns	ns	ns	**	ns	ns	-
C3H/HeOuJ	ns	ns	ns	ns	*	ns	ns
C57BL/6NCrl	ns	ns	ns	****	-	ns	ns

Two way ANOVA test was used for pair wise comparisons between inbred mouse strains at different days (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns not significant). Table reports only days in which significance between inbred mice has been observed.

Table S2. Statistical comparison of change in body weight between inbred mice infected with $5x10^5$ *P. aeruginosa*.

Day	Strain	129S2/SvPasCrl	C3H/HeOuJ	BALB/cAnNCrl
1	A/J	ns	*	ns
2	A/J	ns	**	***
	DBA/2J	***		

Two way ANOVA test was used for pair wise comparisons between inbred mouse strains at different days (*p<0.05, **p<0.01, ***p<0.001, , ns not significant). Table reports only days in which significance between inbred mice has been observed.

Manuscript in preparation

Integrated *in vitro* and *in vivo* genome wide screening strategy for the identification of new virulence targets in *Pseudomonas aeruginosa*

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Abstract

P. aeruginosa remains a major burden in cystic fibrosis, wounds and nosocomial infections due to its high levels of intrinsic and acquired resistance to antibiotics. There is still one third of its genome with no attributed homology to any previously reported sequences with only 6.7% of its genes having a function verified experimentally. Consequently, there is still a lot of information to be uncovered with regards to the mechanisms used by this organism to cause disease with an array of novel antimicrobial targets remaining to be discovered within its genome.

In order to identify novel targets for the treatment and prevention of diseases caused by this bacterium, we have used a genomic approach devised to screen the entire genome of *P. aeruginosa* for novel genes involved in virulence. Using Tn*5* mutagenesis, we have generated a total of 57,360 mutants in *P. aeruginosa* PAO1-L strain. The target screening was performed at different sequential levels. Firstly, mutants were individually tested for reduced swarming as well as pyocyanin and protease production. Then, a selection of those having pleiotropic phenotypes were further examined for attenuation in *Caenorhabditis elegans, Drosophila melanogaster* and, after a further shortlisting, for reduced cytotoxicity on respiratory cell lines.

Finally a total of 8 mutants resulting from these sequential screenings and the selection of genes not attenuated in virulence as controls were verified as potential virulence targets using a combination of a mouse acute infection model, together with cell invasion assays and IL-8 release employing the A549 broncheo-epithelial cell assay. Although the role of some of these genes in the biology of *P. aeruginosa* has already been described (*pvdQ, crc, kdpB* and *bphO*) some of the other open reading frames identified correspond to genes with unknown function (PA2414, PA3613, PA4916 and PA5156). Therefore, this study describes a screening strategy for the identification of novel genes impacting on the virulence of *P. aeruginosa*, which are highly conserved across genomes from *Pseudomonas* spp. and which can be further exploited as novel antimicrobial targets against infections caused by this opportunistic pathogen. This approach has several advantages including expanding the repertoire of bacterial targets, preserving the endogenous microbiota, and applying lower selective pressure, which could result in reduced resistance.

Introduction

Pseudomonas aeruginosa is common throughout the environment and remains one of the most commonly isolated nosocomial pathogen due to its high levels of intrinsic and acquired resistance to antibiotics (Govan and Deretic, 1996). *P. aeruginosa* colonises a large variety of anatomical sites causing a wide range of diseases in humans at various body sites, including the urinary tract, lungs, and burn wounds. Acute *P. aeruginosa* infection in immuno-compromised patients or chronic persistent lung infection in patients with cystic fibrosis (CF) are different at the cellular and organ levels and thus may require distinct treatments. Despite growing evidence about the severity of the diseases caused by *P. aeruginosa*, no drugs with a novel mechanism of action have been introduced. Although extensively investigated in many other pathogens (Shoop *et al*., 2005; Turk *et al*., 2004; Kauppi *et al*., 2003; Hung *et al*., 2005; Lyon *et al*., 2002; Mayville *et al*., 1999; Wright *et al*., 2005; Svenson *et al*., 2001), virulence genes may represent targets for antibiotic discovery that are being explored only recently in *P. aeruginosa (Baer M et al*, Infect. Immun. 2009; Lazar H et al, Antimicrob. Agents Chemother. 2009).

Although there has been a number of genome-wide screening studies to identify *P. aeruginosa* resistance genes and virulence factors (Wei *et al*., 2011; Dötsch *et al*., 2009; Feinbaum *et al*., 2012), the degree to which results of the *P. aeruginosa* virulence screening strategies are shared between multiple hosts remains unclear. A pathogen can experience several radically different “host environments” at various stages of infection (acute or chronic). *P. aeruginosa* is indeed capable of adapting to a wide range of environments and utilizes a broad spectrum of virulence determinants to infect various hosts from plants and insects to humans (Lyczak *et al*., 2000; Lyczak *et al*., 2002). Multiple virulence factors of *P. aeruginosa* include elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, the siderophore pyoverdin, lectins, and biofilm formation (Smith and Iglewski, 2003). The blue pigment pyocyanin, a redox-active phenazine compound that kills mammalian and bacterial cells through the generation of reactive oxygen

intermediates, is regulated through multifactorial mechanisms and hence alteration in pyocyanin production is likely to affect also other virulence determinants (Reimmann *et al*., 1997; Heurlier *et al*., 2004). In addition, swarming plays a key role in virulence and is controlled by several regulatory networks such as the *rhl* and *rsm* systems, which also impact on virulence (Heurlier *et al*., 2004). In *P. aeruginosa*, cell-cell communication (known as Quorum sensing (QS)) is responsible for orchestrating the coordinated production of these virulence factors and promote biofilm maturation (Juhas *et al*., 2005; Williams *et al*., 2007). The *P. aeruginosa* QS system consist of 2 *N*-acyl-homoserine lactone (AHL) regulatory circuits (*las* and *rhl*) linked to an alkyl-quinolone (AQ) QS system. LasR and RhlR are both LuxR transcriptional activators, which activity depends on AHLs synthesized via LasI and RhlI, respectively, to drive virulence gene expression. In various infection models, mutants defective in QS are significantly less pathogenic than their parental strains suggesting that QS systems may be an interesting target for the prevention of the acute and chronic *P. aeruginosa* infections (Pearson *et al*., 2000).

P. aeruginosa possess a 6.3 Mb genome with 32% of its ORFs having no homology to any previously reported sequences. Furthermore, function of only 6.7% of the P. aeruginosa genes has been demonstrated experimentally (www.pseudomonas.com). The rest of the P. aeruginosa genome has been assigned predicted functions, based on homologies to previously characterised genes which remain to be demonstrated. This shows that there is still a large amount of information to be uncovered with regard to mechanisms used by this organism to cause disease, and, an array of antimicrobial targets remains to be discovered within it. In addition, given the intrinsic antimicrobial resistance of *P. aeruginosa* and the need for alternative therapies, there is the possibility that many new targets for novel antimicrobials have yet to be identified.

In order to identify new targets for the treatment and prevention of diseases caused by this bacterium we have used a genomic approach devised to screen the entire *P. aeruginosa* genome for novel virulence genes. Using Tn5 mutagenesis, we have generated a total of 57,360 mutants for PAO1-L. Each mutant was tested for reduced swarming, exo-protease, and pyocyanin production. The selected genes were further validated for reduced toxicity, in a sequential cascade of disease models, including airways cell lines, *D. melanogaster, C. elegans*, and a murine model of acute infection.

Method

Ethics statement

Mice studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

Bacterial strains, growth conditions, plasmids and general DNA manipulation

The bacterial strains and plasmids used are listed in **Table 1S**. *Escherichia coli* and *Pseudomonas aeruginosa* were routinely cultured in Luria Bertani (LB) at 37°C under vigorous shaking (200 rpm). Media were solidified with 1.8% agar (Bacto agar; Difco). The antibiotics were added when required, to final concentrations for tetracycline of 125 μg/ml for *P. aeruginosa* and 10 μg/ml for *E. coli*, and for gentamicin, nalidixic acid, and streptomycin of 20 μg/ml, 15 μg/ml and 500 μg/ml for *P. aeruginosa* and *E. coli*, respectively.

Genomic DNA isolation was performed using a Wizard Genomic DNA purification kit (Promega). Plasmid DNA isolation was performed using a Qiagen Midi Kit (Qiagen). All other DNA manipulation techniques including analysis, digestion, ligation and introduction of DNA into host cells by electroporation were performed as described by Sambrook and Russel (2001).

Transposon mutagenesis

Transposon mutants were generated by mating *P. aeruginosa* PAO1 obtained from D. Haas (Department of Microbiology, University of Lausanne, Switzerland) (referred to as PAO1-L) (Holloway collection) with *E. coli* strain λ*pir* S17.1 carrying pLM1 derived from pRL27 (Larsen *et al*., 2002). *P. aeruginosa* mutants were selected by plating on LB agar containing gentamycin (20 μg/ml), streptomycin (500 μg/ml), and nalidixic acid (15 μg/ml). After incubation at 37°C for 16h mutant colonies were picked using a Flexis (Genomics Solutions, Huntingdon, Cambridgeshire, U.K.) colony-picking robot. Colonies were arrayed into 94-well plates containing 200 μl of 15% glycerol in LB. The microtitre plates were sealed using a gas-permeable membrane (Corning, Gillingham, Dorset, U.K.), incubated at 37°C for 16h and then stored at - 80°C.

Mutant selection

The *P. aeruginosa* mutant strains were screened for alterations in pyocyanin production, swarming motility and/or alkaline protease activity by visual examination with the aid of the colony picker described in the previous section which was programmed to replicate the bacterial cultures from 96-well plates into Omni Trays (Fisher Scientific, Loughborough, Leicestershire, U.K.) containing various solidified media: LB to assess pyocyanin production, swarming agar, or LB containing 5% (w/v) of skimmed milk for determination of protease activity.

Transposon insertion location

To determine the transposon insertion point in the chromosome of the selected Tn*5* mutants, total genomic DNA was isolated and digested with *EcoR*I, which does not cut pLM1. Digested genomic DNA fragments were recircularised and, upon electroporation in *E. coli* λ*pir* S17.1, selected for gentamicin resistance. DNA insert regions flanking the Tn*5* were sequenced using primers tpnRL17-1 (5'-AACAAGCCAGGGATGTAACG-3') and tpnRL17-2 (5'-CAGCAACACCTTCTTCACGA-3'), at the Biopolymer

synthesis and analysis department, Queens Medical Centre, Nottingham, U.K. DNA sequence analysis was performed using software packages provided by the National Centre for Biotechnology Information BLAST network server.

C. elegans and _D. melanogaster_ virulence assays

To investigate the level of virulence attenuation of the selected mutants C*. elegans* and *D. melanogaster* disease models were employed. For the former, the *C. elegans* strain DH26 was used in slow killing assays. Worms were synchronized into L4 larval stadium by egg preparation, which was followed by incubation of isolated eggs on *E. coli* OP50 feeding plates at 20°C for around 76 hours. Subsequently, L4 larvae were transferred onto lawns of the *P. aeruginosa* mutant strains grown in the 6-well plates (approximately 30 worms per well) and incubated at 25°C. The surviving worms were counted after 24, 48 and 72 hours with the aid of a Stereolupe Stemi SV 6 (Zeiss, Goettingen, Germany).

The pathogenicity of the mutants was determined from the survival rates of *C. elegans* in three independent replicates. The *D. melanogaster* disease model was used as previously described (Apidianakis & Rahme, 2009). *D. melanogaster* Canton-S wild type flies were bred on standard cornmeal sucrose medium at room temperature. 2-9 days old male and female flies were used as infection hosts. Bacterial strains were grown in LB to an OD_{600} of 1.0. The cells were harvest, washed and adjusted with 10 mM $MgSO_4$ to an OD_{600} of 2.0. Flies were anaesthetized with ether and bacterial suspensions were inoculated into the thorax using a tungsten needle. For each trial, triplicate sets of 15 flies were used and the experiment was performed in duplicate. The negative control flies were inoculated with a 10 mM $MgSO_4$ solution. If more than 5 flies died because of injury caused during pricking, the experiment was repeated. The pricked flies were incubated at 26°C and counted every hour between 11h to 22h post infection.

In silico analysis

Comparative protein sequence analysis was carried out against the six sequenced genomes (PA14, LESB58, PA7, 2192, C3719 and PACS2) of *P. aeruginosa* and against *E.coli* K12 and *B. cenocepacia* J2315 in order to check presence and conservation of our candidates in other *P. aeruginosa* genomes and in other bacterial species. The BLASTP program, present on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), was used for homology analysis. Analysis was done by aligning the amino acid sequence of PAO1 against other genomes.Genes identified in our screen were then checked for presence in the set of virulence-related genes annotated in the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/) (Chen L *et al*., 2005).

Cell cultures and cytotoxicity assay

A549 alveolar epithelial cells ($1.2x10^5$/ml) were seeded on chamber slides and incubated with 500 µl cell culture medium DMEM per well over night until they reached a cell number of $2.4x10^5$/ml. *P. aeruginosa* strains were cultured overnight in 5ml Trypton Soy Broth (TSB). From the overnight culture a fresh culture in 5ml TSB (optical density, OD 0.05) was prepared and incubated at 37°C until a optical density of 0.5 (4-$5x10^8$ CFU/ml) was reached. This culture was centrifuged (2700 x g) at 4°C for 15 min and the culture supernatant was harvested and filtered through a sterile filter (pore size 0.22 µm). From the culture supernatant 500 µl were put into the wells of a chamber slide containing A549 cell monolayer and incubated for 3h at 37°C 5% CO_2. The bacterial cell pellet was resuspended in 10 ml cell culture medium DMEM without antibiotics, centrifuged (2700 x g) at 4°C for 15 min and resuspended in 1 ml DMEM. This bacterial suspension was diluted with DMEM to obtain a bacterial cell number of $2x10^5$ CFU/ml. In order to block unspecific binding of the bacteria, bovine serum albumin (1%) in DMEM was put into the wells the of a chamber slide containin A549 cell monolayers and incubated for 30 min at 37°C 5% CO_2. From the bacterial suspension 500 µl were put into the wells of the chamber slide and incubated for 3h at 37°C 5% CO_2. After incubation of the culture supernatant and bacteria cells with the A549 cells the culture supernatant was removed and the cells were stained with 200 µl solution, containing 4 µl PI and 4 µl Syto 13 and 2 ml phosphate buffered saline (PBS), for 10 min at 37°C in a dark chamber. The cells were washed three times with 500 µl PBS and fixed immediately with 5% glutaraldehyde (Sigma) over night at 4°C. The cells were washed, the chamber was removed and the slide was embedded in Fluorescence mounting medium (Dako). The cells were analysed with the Zeiss Axioplan Fluorescence Microscope and the Axio Vision Programm (Zeiss). Five digital pictures were taken from each well at a magnification of 200. Dead cells were calculated by counting the yellow cells (dead cells) resulting from co staining of PI and Syto 13 compared to green cells (living cells) only stained with Syto 13.

Cell culture and invasion assay

The A549 (human type II pneumocytes) cell line was purchased from ATCC CCL-185 and cultured as described (Pirone *et al*., 2008). Bacteria invasion assay was performed using Polymvxins B (100 µg/ml) (Sigma) protection assay with minor modifications (Lorè *et al*., 2012). *P*. *aeruginosa* strains, grown to the mid-exponential phase, were used to infect cell monolayers at a 100:1 multiplicity of infection for two hours. The monolayers were washed with PBS, treated with antibiotic for two hours, washed, lysed with H_20 and plated on TSB-agar plates (Difco).

IL-8 secretion

IL-8 was determined in supernatants collected from the cell cultures described above using an ELISA kit (Biosource Europe). According to the manufacturer the sensitivity of the assay is less than 0,7 pg/ml. Values were normalized to 10^6 cells; results were expressed as mean +/- SD.

Mouse model of acute *P. aeruginosa* infection

C57Bl/6NCrlBR mice (20-22 gr) were purchased from Charles River Laboratories, Italy. Mice were housed in filtered cages under specific-pathogen conditions and permitted unlimited access to food and water.

Prior to animal experiments, the *P. aeruginosa* mutant strains were grown for 3h to reach mid exponential growth phase. Next, bacteria were harvested, washed twice with sterile PBS and the OD of the bacterial suspension adjusted to an OD 600 nm. Planktonic bacteria were resuspended in sterile PBS to the desired dose for infection of $5x10^6$ CFU/mouse. Mice were anesthetized and the trachea directly visualised by a ventral midline incision, exposed and intubated with a sterile, flexible 22-gr cannula attached to a 1 ml syringe accordingly to established procedures (Bragonzi *et al*., 2009). A 60 µl inoculum of $5x10^6$ CFU were implanted via the cannula into the lung, with both lobes inoculated. After infection, survival was monitored twice a day for up to four days. Mice that lost >20% body weight and had evidence of severe clinical disease, such as scruffy coat, inactivity, loss of appetite, poor ambulation, dehydration or painful posture, were sacrificed by CO_2 administration before **termination** of the experiment. In another group of mice, the lungs were excised and processed for histopathology.

Histological analysis

Mice were sacrificed by CO_2 administration after 24h of infection, lungs were removed en bloc and fixed in 10% buffered formalin at 4°C for at least 24h and processed for paraffin embedding. Longitudinal sections of 5 µm from the proximal, medial and distal lung regions were obtained at regular intervals using a microtome.

Sections were stained with haematoxylin and eosin according to standard procedures. Areas of inflammatory cell infiltration and tissue preservation (normal histology) were quantified using Image J software (National Institutes of Health) and reported as a percentage of total area.

Statistical analysis

Results are presented as mean ± SEM. Statistical calculations and tests were performed using Student's paired t-test, and Log rank Mantel-Cox test in order to determine the significance of differences in means between pairs.

Results

Identification of *P. aeruginosa* transposon mutants with pleiotropic phenotypes

To identify novel genes involved in the virulence of *P. aeruginosa* and which can be exploited as antimicrobial targets, a strategy involving a multistep sequential approach shown in **Figure 1A** was used. This started with the construction of a Tn*5* mutant library in *P. aeruginosa*. To perform this mutagenesis

pLM1 was used due to the high transposition efficiency of this Tn5-based vector in *P. aeruginosa* (Larsen *et al*., 2002). We used PAO1-L as the strain of choice since we did not identify some of the alterations found in other PAO1 strains such a large genomic deletion from PAO1-N (Prochnow *et al.*, in preparation). A total of 57,360 mutant strains (598 plates of 96 individual mutants) were obtained by conjugation. We estimated that around 60,000 mutants of PAO1-L needed to be generated to have 95% chance of disrupting any given gene of 327 bps according to Liberati et al (2006). To enable the use of a high throughput (HTP) screening for the isolation of mutants with attenuated virulence, simple bioassays were designed using a robotic system (**Fig. 1A**). All the mutants were tested individually in qualitative manner for attenuations in swarming, the loss of the blue pigment pyocyanin in liquid media, and reduction in protease production using 5% skimmed milk agar plates. All mutants with alterations on one of these three phenotypes were then rescreened for attenuation of the other two phenotypes (**Fig. 1A**). A total of 404 mutant strains showing pleiotropic phenotypes (**Fig. 1B**) were selected for the second stage of screening using the *C. elegans* and *D. melanogaster* infection models.

Impact of pleiotropic selected mutants on virulence attenuation using the *C. elegans* and *D. melanogaster* disease models

The 404 mutants showing pleiotropic phenotypes from the initial Tn*5* library screening were further tested in a second stage screening. This was based on the use of *C. elegans* and *D. melanogaster* as infection hosts to identify attenuated mutants. The aim was to further shortlist these mutants prior to evaluating their cytotoxicity in eukaryotic cells lines (**Fig. 1A** and **1B**).

From a total of 404 transposon mutants tested, 108 were found to be attenuated in at least one of the infection models. All 108 mutants were further characterized using quantitative assays for alteration of swarming, pyocyanin and alkaline protease (as described in the supplementary materials and methods). In this characterization, we included also a total of six mutants (PA5156, PA4265, PA3613, PA3448, PA0534 and PA0425) that were not attenuated either in *C. elegans* or in *D. melanogaster* as part of the validation of our screening strategy. It is important to note that none of the mutants showed growth defects (data not shown). This screening identified a total of 47 mutants attenuated in all three virulence traits, 51 mutants in two traits and 16 mutants in one trait. The insertion sites of the 108 transposon mutants attenuated in *C. elegans* or *D. melanogaster* and the six "control" mutants not attenuated in these models were identified with a view to eliminate mutant redundancy (see materials and methods). These resulted in a reduction of the numbers of mutants with attenuated virulence in at least one non-mammalian model to 67 with unique insertions in ORFs, representing 1.3% of the entire genome coding sequence, plus 4 within intergenic regions, (Stover *et al*., 2000) (**Fig. 1A** and **1C**). **Table 1** shows the detailed results obtained for 71 mutants selected, including also 6 mutants without attenuation in non-mammalian models as described before.

Functional diversity of the genes involved in virulence

Analysis of the predicted proteins encoded by the 67 ORFs with unique Tn5 insertions and attenuated in virulence revealed that these proteins are involved in various cellular functions (**Table 1**). These proteins were grouped into seventeen functional gene classes, including energy, amino acid and nucleotide metabolisms, transcriptional and post-transcriptional regulation, motility and adaptation, secretion and transport of small molecules, enzymes as well as conserved hypothetical proteins of unknown functions (**Table 1**). There was a wide spread distribution for the predicted functional category of the mutated genes, with the highest abundance corresponding to 24 genes with unknown function followed at a distance by 7 genes involved in transport of small molecules. (**Table 1** and **Fig. 2**). For all the genes identified only 20 had been previously demonstrated to be involved in virulence determinants in *P. aeruginosa*. The various functions of these 20 genes, including antibiotic resistance (*parR*, *phoR*, *mexA* and *rpoS*), biofilm formation (*pelG*, *pslJ*, *mucP* and *cupA3*), virulence factors delivery (*tssA1*, *pilD*, *xcpT* and *plcH*), regulation (*pqsA*, *pqsC*, *pvdQ*, *crc*, *pilU* and *ptsP*), and metabolism (*bphO* and *nagE*) (**Table 1**) support the robustness of the screening strategy used. In order to validate our screening method, we compared the 67 genes identified with two unbiased screens based on negative and positive signature tagged mutagenesis (STM) selection methods. The negative selection approach applied to animal models of short-term acute infection identified *P. aeruginosa* mutants with attenuated virulence, while the positive approach searched for *P. aeruginosa* pathogenicity-adaptive mutations in a murine chronic respiratory infection models (Potvin *et al*., 2003; Bianconi *et al*., 2011). Our screen identified six genes that were already selected by negative-STM selection (ORFs PA3110, PA4098, PA4489, *tssA1*, *cupA3*, *pilD*) and one by positive-STM selection (*nqrB*) (Potvin *et al*., 2003; Bianconi *et al*., 2011) (**Table 1**).

We compared the results obtained in *C. elegans* in our work with the large set of virulence genes identified by Feinbaum and colleagues (Feinbaum *et al*., 2012) using the same non-mammal model. Our screen identified two genes selected also in Feinbaum work (*ptsP*, *gshA*) (**Table 1**). In particular, *pstP* was previously demonstrated to be a virulence factor in *C. elegans* (Tan MW *et al*., 1999) (**Table 1**).

In silico and comparative genomic analysis of selected targets

Among the 67 targets identified, with the exclusion of the intergenic regions, 18 belong to the class of confidence 1 and are fully characterized in *P. aeruginosa*, 14 belong to class 2 and are similar to protein characterized in other species, 12 belong to class 3 and 23 belong to class 4, these last targets have an hypothetical function or they are completely unknown. Among the targets, six (*tssA1*, *pilU*, *plcH*, *pvdQ*, *mucP*, *pilD*) are known virulence factors annotated in the VFDB (Virulence Factors Database).

Pseudomonas aeruginosa isolates have a highly conserved core genome representing up to 90% of the total genomic sequence with additional variable accessory genes, many of which are found in genomic islands or islets. Exchange of loci encoding virulence genes may occur by mutations and homologous recombination

which occur frequently in *P. aeruginosa* particularly in CF patients. To address the global relevance of these virulence factors, a comparative protein sequence analysis was carried out against six sequenced genomes (PA14, LESB58, PA7, 2192, C3719 and PACS2) of *P. aeruginosa* and against *E. coli* K12 and *B. cenocepacia* J2315 in order to check presence and conservation of our candidates in other *P. aeruginosa* genomes and in other bacterial species (**Table 2S**). The majority of the selected targets are conserved in all the six *P. aeruginosa* genomes analysed, eight are conserved in five genomes, one (*tufA*) is conserved only in four genomes and one (PA0823) only in three genomes. Ten targets are highly conserved in *B. cenocepacia* J2315, with a homology higher than 60%, whereas 22 have no or very poor (<20%) homology. *tufA* has a very high homology (>80%) with *E. coli* K12, 26 targets are rather conserved with a homology between 41% and 60%, while the majority of the targets (35) are poorly conserved, with a homology between 20% and 40%.

Nine targets (*tssA1*, *ptsP*, *cupA3*, *nqrB*, *pilD*, *gshA*, the ORFs PA3110, PA4098, PA4489) were previously identified in other *in vivo* large genomic screenings as factors strictly related to *P. aeruginosa* virulence, as hinted in the previous paragraph (Bianconi *et al*., 2011; Potvin *et al*., 2003; Winstanley *et al*., 2009; Feinbaum *et al*., 2012).

Complete homology analysis of the selected targets is present in **table S2** (supplementary material).

Validation of targets selected for murine acute infection

For the next stage in the evaluation of the strategy outlined in this manuscript for the identification of virulence targets, eight mutant strains attenuated in one, both or none of the *C. elegans* and *D. melanogaster* disease models were selected for further validation analysis using: (i) quantitative *in vitro* analysis for attenuation of virulence traits, (ii) *C. elegans* and *D. melanogaster* killing curves, (iii) cell invasion and (iv) cytokine production assays. The selected mutants were *pvdQ* and *crc* as they were attenuated in all phenotypes, disease models and cytotoxicity assays (**Table 1**), the mutants in *kdpB*, *bphO*, PA2414 and PA4916 as they were attenuated in one of the disease models and showed differences *in vitro* virulence assays, and finally PA5156 and PA3613 as they did not show attenuation in the disease models but showed again different levels of attenuation in the other virulence assays (**Table 1**).

Analysis of the 8 selected mutants for surface motility, biofilm formation, and their ability to produce elastase and pyoverdine showed in all of them a reduction in the production of elastase and biofilm formation. However PA3613 did not show any reduction in pyoverdine, or any of the motility tests used as anticipated from the results shown in (**Fig. 1S**).

The impact of the 8 selected mutations on *D. melanogaster* and *C. elegans* was confirmed by following hosts survival rate over a period of 24h for *C. elegans* and 72h for *D. melanogaster* (**Fig. 3**).

The killing curves confirmed the results shown in Table 1, except that PA1634 was found to be more pathogenic for *C. elegans* than in the initial screen (**Fig. 3A**). Notably, the *crc* mutant was the most attenuated strain in both infection models (**Fig. 3**).

In accordance with the previous outcomes of the sequential screening cascade approach we challenged A549 cells with the eight mutants in order to validate the attenuation of the candidates in terms of invasion capacity and secretion of the pro-inflammatory cytokine IL-8 (**Fig. 4**). Seven out of the eight mutants selected were significantly attenuated in term of invasion capacity (PAO1-L wild type vs *kdpB* $p<0.001$ *pvdQ* $p<0.001$, *bphO* $p<0.001$, *crc* $p<0.001$, PA2414 $p<0.05$, PA4916 $p<0.01$, PA5156 $p<0.001$) and six mutants were attenuated in IL-8 release (PAO1-L wild type vs *kdpB* $p<0.05$, *pvdQ* $p<0.05$, *bphO* $p<0.01$, PA5332 (*crc*) $p<0.05$, PA2414 $p<0.05$, PA5156 $p<0.01$, and) in comparison to the wild type PAO1-L (**Fig. 4A** and **B**).

Impact of selected mutants in the mouse model of acute lung infection

Combining all the results from *in vitro* screening and virulence assays, 8 Tn*5* mutants (*kdpB*, *pvdQ*, *bphO*, *crc*, PA2414, PA3613, PA4916, PA5156) were selected in order to test differences in lethality in the mammalian host using a murine model of acute lung infection (**Fig. 5**). First, escalating doses ranging from $5x10^5$ to 10^7 CFU of wild type PAO1-L were intratracheally injected in C57BL/6NCrlBR mice to determine the relative range of susceptibility (**Fig. 2S**). The first lethal dose was $5x10^6$ CFU/lung (**Fig. 2S**). C57BL/6NCrlBR mice were then infected by intra-tracheal injection with $5x10^6$ CFU of planktonic wild type PAO1-L and transposon mutants and survival monitored up to 96h (**Fig. 5**). While wild type PAO1-L was totally lethal within 36h, the lethality of transposon mutants was significantly lower and temporally shifted (**Fig. 5A**). Five of these mutants were significantly attenuated in inducing mortality when compared to wild type PAO1-L (wild type PAO1-L vs *pvdQ*, *bphO*, *crc* $p<0.001$; wild type PAO1-L vs PA4916 and PA5156 $p<0.05$; Mantel-Cox test) (**Fig. 5A**). Among these mutants attenuated, it should be noticed that both PA4916 and PA5156 were not attenuated in *C. elegans* and in other models. In addition, mutants attenuated in *C. elegans* were not attenuated in the mouse model. Overall, these results suggest a host-specific response to *P. aeruginosa* and indicate the necessity to test a selection of mutants in the mouse model, as the nearest to the human host.
Furthermore, histopathological analysis (**Fig. 5B**) showed that wild type PAO1-L strain (**Fig. 5C**) induced slightly highest inflammation and haemorrhage in comparison to the *crc* (**Fig. 5D**) and *pvdQ* mutants (**Fig. 5E**). The area infiltrated by inflammatory cells was significantly higher in the lungs infected by wild type PAO1-L in comparison to the mutant *pvdQ* (wild type PAO1-L and *crc* vs *pvdQ* $p<0.001$, Mann Whitney test) (**Fig. 5F**).

Conclusions

In this study, we have described a new screening strategy combining an *in vitro* testing for reduced production of virulence factors and validation in a cascade of *in vitro* and *in vivo* virulence models including airways cells, *C. elegans*, *D. melanogaster* and a mouse model of acute lung infection. We have isolated a total of 404 independent pleiotropic mutants (attenuated in two or more virulence traits). All 404 mutants were screened for reduced virulence in the two non-mammalian infection models. Mutants that showed reduced virulence in at least one of these models were tested for their reduced cytotoxicity on A549 alveolar epithelial cells. Consequently, a total of 114 mutants were selected for identification of the inserted gene, leading to the identification of 67 virulence genes. Through our screening, we were able to isolate a large number of mutants with insertions in genes already demonstrated to be required for *P. aeruginosa* virulence in nematodes and mammals, including genes involved in antibiotic resistance, biofilm formation, virulence factors production, metabolism and quorum sensing regulation. Bioinformatics was employed to select targets broadly conserved among the desired bacterial spectrum, but absent or evolutionary distant in eukaryotes. A large proportion of the identified genes were highly conserved within *P. aeruginosa* and the two Gram negative pathogens *E. coli* and *B. cenocepacia*. Six of the 67 identified genes are in the database of *Pseudomonas* related virulence (VFDB) and 9 genes were previously identified as related to *P. aeruginosa* virulence (Bianconi *et al*., 2011; Potvin *et al*., 2003; Winstanley *et al*., 2009; Feinbaum *et al*., 2012).

A comparison with the recently published *C. elegans* screen from Feinbaum et al. (2012) showed that there is little overlap between the two screening strategies. Both screens have identified *orfs* from *gshA-B* and *kpdB-D* operons. In their paper, Feinbaum and colleagues hypothesised that *kdpD* and *gshA-B* could be required for *Pseudomonas* adaptation in the host since the loss of membrane integrity of a *kdpD* mutant and the susceptibility of a *gshA* mutant to pH variation may affect bacterial cell survival (Feinbaum *et al*., 2012). Similar to the *C. elegans* screen, we identified a number of genes that are involved in adaptation to environmental stress by playing a protective role. Those include a large number of metabolic genes involved in the generation and transport of precursor metabolites and energy (*fdhE*, *ptsP*, *xdhB*, bphO, nqrB, nagE and PA2414), and genes involved in DNA synthesis and modification (*smpB*, *pdxA*, PA3867, PA3950 and PA4282) (**Table 1** and **Fig. 3**). Interestingly, genes playing a part in amino acid synthesis and metabolism were identified (*pauB1* coding for a D-amino acid oxidase, *hisD* coding for a histidinol dehydrogenase, PA3139 coding for a threonine-phosphate-decarboxylase). Amino acids are available to bacterium in the lung environment and a more efficient amino acid acquisition could be related to the biosynthetic cost of producing each metabolite. The identification of a number of mutants corresponding to biofilm formation (*psl*, *mucP* and *pelG*), type IV and VI secretion systems (*xcpT*, *pilD* and *tssA1*), and stress response (*phoR*, *rpoS* and *parR*) also illustrate their importance for survival of *P. aeruginosa* in the "host environment". With respect to the virulence screening assays, one explanation for why a majority of the

mutants identified in this study correspond to metabolic and stress adaptation functions may be the growth conditions (e.g. low aeration) applied during the screening.

Our screening approach favoured the identification of mutants attenuated in all virulence factors. However, the observation that six mutants (PA5156, PA4265, PA3613, PA3448, PA0534 and PA0425) were selected for their pleiotropic phenotypes but were not attenuated in the disease models *C. elegans* and *D. melanogaster* demonstrates the importance of the simultaneous utilization of multiple screening strategies. In addition, while we have found accordance between presence or absence of attenuation for some targets in *C. elegans* and in the murine model (*kdpB*, *pvdQ*, *bphO*, *crc*, PA3613), PA2414 was attenuated in *C. elegans* but not in mice and PA4916 and PA5156 were attenuated in the mouse model of acute infection, but not in *C. elegans*. Other incongruities were observed between the results obtained in mice and those from other screenings. Our data show that identification of virulence genes carried out mainly *in vitro* does not imply that they are relevant for their pathogenesis *in vivo*. In the context of infections caused by *P. aeruginosa* virulence is mediated by a wide variety of trans-acting regulators that sense the environment and the physiological state of the cell and adjust the transcription of specific genes changing their phenotype significantly. The use of non-mammalian infection models has several downsides, as the temperature for the cultivation of the nematode that affects the expression of certain virulence factors, the absence of the target organ and the lack of specific receptors or pathways. However, although rodents are the first choice for understanding infectious diseases in human, screening a large amount of targets in mouse models is unfeasible. Despite the downsides described above, non-mammalian models remain useful surrogate hosts. In addition to being largely used to identify virulence factors, *C. elegans* has been exploited to study responses to infection as well as to compare the virulence of clinical and environmental isolates (Monk *et al.*, 2008). Furthermore, pathways conserved in vertebrates characterize *Drosophila* response to pathogens, and mammalian and *C. elegans* innate immune defenses (Ferrandon *et al.*, 2007;. Engelmann *et al.*, 2010). Therefore, a multifaceted bottleneck approach, by using a sequential cascade of models to provide the rationale and the proof-of-concept, is essential for the characterization and validation of a large number of bacterial target candidates. However, the approach employed in this work, although representing a step forward in understanding *P. aeruginosa* pathogenesis, does not totally reflect the complex situation taking place in infected patients. Several evidences are transforming the view of infectious diseases from strictly pathogen-centric to the one incorporating host environmental and genetic determinants that modulate immune response. Thus, a wide combination of different validation models including mouse model, as the nearest to the human host, are necessary to test mutant strains of *P. aeruginosa*, although not thoroughly exhaustive.

The selection of virulence mutants based on pyocyanin deficiency could be another limitation to the screening strategy. It is still controversial whether this phenazine correlates with worse conditions in CF patients (Carlsson *et al*., 2011; Hunter *et al.*, 2012; Rada *et al.,* 2013). In addition, Bianconi and colleagues

(Bianconi *et al.*, 2011) have found that a PilY1 mutation favors *P. aeruginosa* persistence. The absence of PilY1 leads to a reduction of pyocyanin, which suggests that inhibiting pyocyanin synthesis could favor bacterial persistence thus leading to chronic infections in CF.

Growing knowledge about the physiology of pathogens and hosts over the last decade has boosted the molecular understanding of the factors that promote bacterial pathogenicity in the course of the infection process. Anti-virulence signalling strategies (Rasko *et al.*, 2010; Brotz-Oesterhelt *et al.*, 2010; Fernebro, 2011) may specifically interfere with the ability of the bacteria to recognize host signals and/or activate specific virulence traits that are needed to establish infection. By preventing the expression or activity of virulence traits, the bacteria are less able to colonize the host. Broad-spectrum antibiotics are not always the best choice, especially not when considering the commensal flora and the risk for opportunistic infections. The removal of species, or alteration of the balance of species in a community as complex as the gastrointestinal or airways microbiota, could result in detrimental effects for the host. Targeting bacterial virulence is attractive since it is specific toward pathogenic bacteria and spares the commensal flora. In addition, as this strategy does not directly kill the bacteria, there is presumably less evolutionary pressure for the development of resistance than with traditional antibiotics. This inhibition could also allow the host immune system, including the normal microbiota, to prevent bacterial colonization or clear any established infection. Some concerns should be considered about anti-virulence therapy for *P. aeruginosa* infections. *P. aeruginosa* virulence is a highly complex, multifactorial process requiring the coordinated activity of many bacterial gene products which can be widely different depending on the infection is established. The idea of a core set of virulence factors common to all infection models is unlikely. The spectrum of virulence factors that play a role in a given host model depends on a wide variety of factors including the characteristics of the site of infection, the type of the immune response and the phase of infection, and even host behavior. Virulence factors playing a role in distinct types of *P. aeruginosa* infection in humans could be different, implicating a great effort to personalize the treatment and thus posing a challenge for the development of new therapeutics.

Another concern deals with the anti-virulence approach in CF chronic infection. It has been shown that long-term colonization of the CF host is maintained by *P. aeruginosa* patho-adaptive lineages, which are clonal with the initially acquired strain and carried phenotypic variants (Smith *et al.*, 2006). A number of genetic mechanisms are responsible for generating clonal variants in *P. aeruginosa*. Most CF strains consistently acquire common mutations in virulence genes including in motility genes, in quorum-sensing regulator, in the type-III secretion system, in the multidrug-efflux pump and in mutator phenotypes (Bragonzi *et al.,* 2009; Alcalá-Franco *et al.*, 2012). Interestingly, virulence factors required for the initiation of acute infections were selected against during chronic infection. This indicates reduced virulence of the late strains with regard to their ability to provoke acute infection. This evolutionary scenario is similar to that of the genomes of other pathogens. Genetic loss-of-function mutations confer indeed enhanced

fitness of the pathogen in a host-associated environment as shown for other pathogen (Akopyants *et al*., 1995; Moxon *et al.*, 1978; Sokurenko *et al.*, 1998). Loss of virulence factors thus represents a strategy used by the pathogen to adapt to the environment and persist in the host (Bianconi *et al.*, 2011). This raises the question whether anti-virulence therapy could favor *P. aeruginosa* fitness to the CF lung leading to chronic infections. One possibility could be to target virulence genes only during acute phases of *P. aeruginosa* infection in CF patients, aiming to the eradication by the host immune system.

Anti-virulence strategies have still been poorly exploited to treat *P. aeruginosa* infection and they were directed mainly towards known virulence targets, namely PcrV, a protein of the T3SS, and the O-polysaccharide moiety of *P. aeruginosa* serotype O11 (Baer *et al.*, 2009; Lazar *et al.*, 2009). This could be due to the fact that large parts of *P. aeruginosa* genome, in which an array of unknown antimicrobial targets may be hidden, are still unexplored, probably for the lack of genetic and functional tools for large-scale screening. In this study, out of 67 genes, our multifaceted screening approach identified 22 new virulence genes with still unknown function, 3 of which (PA2414, PA4916 and PA5156) were validated in murine model of acute infection. We identified a large number of unstudied genes that are involved in virulence and that might turn out to be targets of clinical relevance. Furthermore, the observation of independent selection of multiple strains with the same mutation indicated that our screen was effective in determining virulence deficiencies.

In summary, by using a genomic approach devised to screen the entire *P. aeruginosa* genome for novel virulence genes and a multifaceted approach based on a sequential cascade of models for the validation step, we have identified several novel virulence genes. These genes should be further investigated for their function and could represent interesting targets for an innovative anti-virulence approach to *P. aeruginosa* infections.

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LEGENDS

Table 1. List of the genes involved in *P. aeruginosa* PAO1-L virulence. The attenuated phenotype is indicated by a red box while not attenuated phenotype is indicated by a blue box. Genes are ordered according to their increasing locus tag-PA number in PAO1. The number of transposon insertions, the class of confidence (1: function experimentally demonstrated, 2: function of highly similar gene experimentally demonstrated in other organism, 3: function proposed based on presence of structural feature, 4: homolog of previously reported genes of unknown function), if targets are annotated as known virulence factors, the homology with other than PAO1 *P. aeruginosa* genomes and genome of other gram-negative species and the identification of the gene in other screening are also indicated. The eight genes which were examined for their involvement in pathogenesis using the murine acute infection model are highlighted in grey. [&] genes annotated as virulence genes in the Virulence Factor Database (http://www.mgc.ac.cn/VFs/); * numbers indicate the numbers of checked *P. aeruginosa* genome in which the target is conserved, homology >80%; [#] UH unknown homology, 0 homology <20%, 1 homology between 20-40%, 2 homology between 41-60%, 3 homology between 61-80%, 4 homology >80%; [~] Infection assays were carried out as described in the Material and Methods. Mutant strains were considered to be attenuated when more than 40% of the flies survived 24 h post infection; [$] Bacteria were grown for 3 h and washed bacteria were incubated with A549 cells. Culture supernatants were obtained from bacteria grown for 18 h and incubated with A549 cells. Cytotoxicity lower than 4% was defined as highly decreased cytotoxic capacity. Three independent experiments were performed with triplicates. Values represent mean ± standard deviation (SD). The Student's t-test was used.

Figure 1. Selection of *P. aeruginosa* transposon mutants with pleiotropic phenotype. A) Screening strategy leading to target identification. A transposon library of *P. aeruginosa* PAO1-L comprising 57,360

mutants were screened for attenuated virulence *in vitro*, including production of protease and pyocyanin and swarming motility. A total of 404 transposon mutants having a pleiotropic phenotype were further tested in a sequential cascade of disease models including A549 epithelial cell line, *C. elegans* and *D. melanogaster*, resulting in the selection of 114 to be mapped. A total of 8 mutants which were attenuated in both, one or none of the disease models were evaluated in a mouse model of acute infection to provide rational and proof of concept. **B**) Summary of the results of the *in vitro* screening**.** A total of 404 mutants having pleiotropic phenotypes (impaired in protease, pyocyanin and swarming motility) were further examined *in vivo***. C**) Summary of the results of the transposon library.

Figure 2. Proportion of genes (sorted by function) represented in the list of 67 candidate targets.

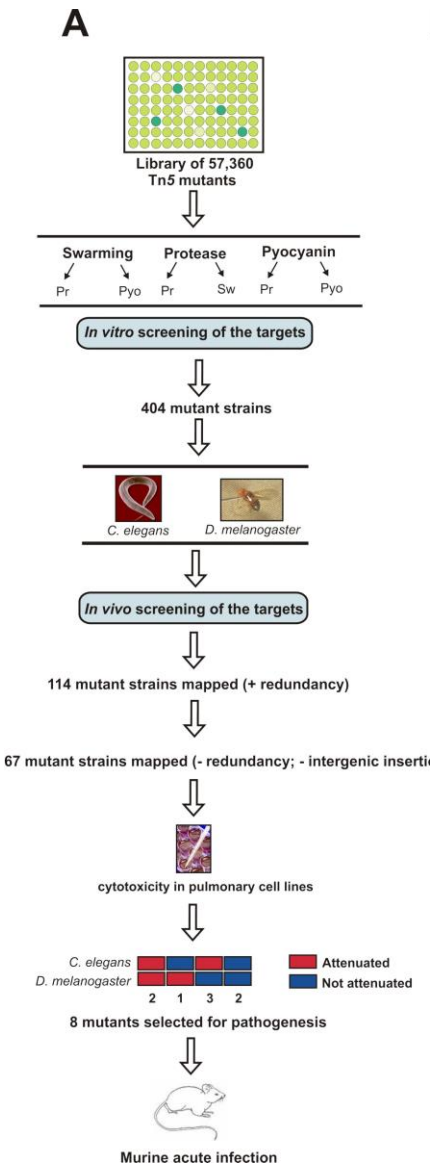
Figure 3. **Virulence of *P. aeruginosa* PAO1-L and investigated transposon mutants in *C. elegans* and *D. melanogaster* disease models. A**) Lethality curves in *D. melanogaster* disease model. **B**) Lethality curves for 8 investigated mutants in the *C. elegans* disease model. The results presented here show the mean values and standard errors calculated from three independent experiments.

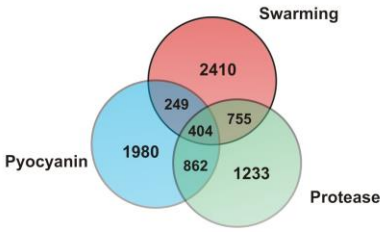
Figure 4. Virulence of *P. aeruginosa* PAO1-L and its transposon mutants in A549 alveolar cells. A total of 8 transposon mutants of *P. aeruginosa* PAO1-L were screened for their ability to invade the bronco-epithelial cell line A549 and to induce IL-8 release. **A**) Internalization of the bacterial cells was tested using an antibiotic exclusion assay. A total of seven mutants out of eight (*kdpB*, *pvdQ*, *bphO*, *crc*, PA2414, PA4916, PA5156) were significantly attenuated in their capacity to invade A459 in comparison to wild type strain. **B**) IL-8 release was measured by ELISA in the supernatant of cells infected with the transposon mutants. IL-8 release was significantly attenuated in the supernatants of A549 cells after infection with six transposon mutants (*kdpB*, *pvdQ*, *bphO*, *crc*, PA2414, PA5156) in comparison to wild type PAO1-L. Data, from three independent experiments performed in triplicates, are expressed as mean +/- SD. *p<0.05, **p<0.01, ***p<0.001 in the Student's t-test.

Figure 5. Virulence of *P. aeruginosa* PAO1-L and a selection of transposon mutants in a mouse model of acute lung infection in C57Bl/6NCrl mice. Mice were intratracheally injected with $5x10^6$ CFU of PAO1-L wild type or Tn5 mutants. **A**) Survival was monitored up to 96 hours. Five of these mutants were attenuated in inducing mortality when compared to the wild type, *bphO*, *pvdQ*, *crc*, PA4916 and PA5156. **B**) Lung histopathology was performed after 24h from infection for wild type PAO1-L , *pvdQ* , and *crc*. Infiltrated area was evaluated based on histopathology. **C**) Evaluation of the inflammatory score showed that *crc* and *pvdQ* were slightly attenuated in their capacity to induce leukocytes recruitment and lung damage in comparison to the wild type. Two independent experiments were pooled. Statistical analysis was calculated

for pair wise comparisons between wild type and mutant strains. *p<0.05, **p<0.01, ***p<0.001, Mantel-Cox test.

Figure 1





C

Category	Number
Mutants arrayed	57,360
Identical insertion location	71
Insertion inside ORF	67
Insertion between ORF	4
Insertion identified in other screenings	9
Mutants scored for colony phenotype	404
Mutants tested in at least one of virulence model	108
Mutants tested for IL-8 production, cell invasion	8
Mutants tested in mice	8

Figure 2

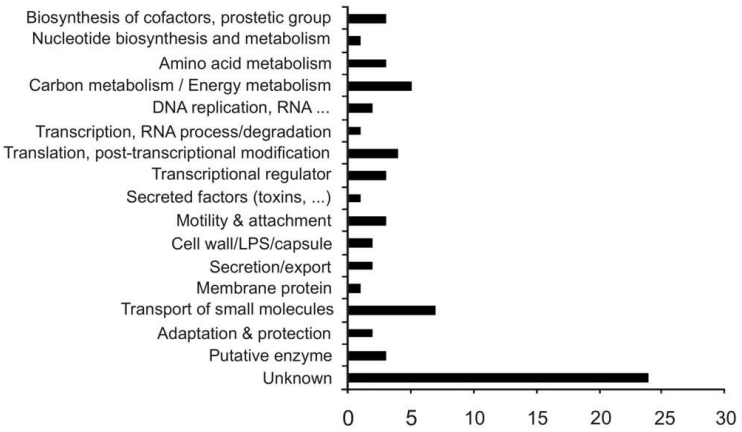


Figure 3

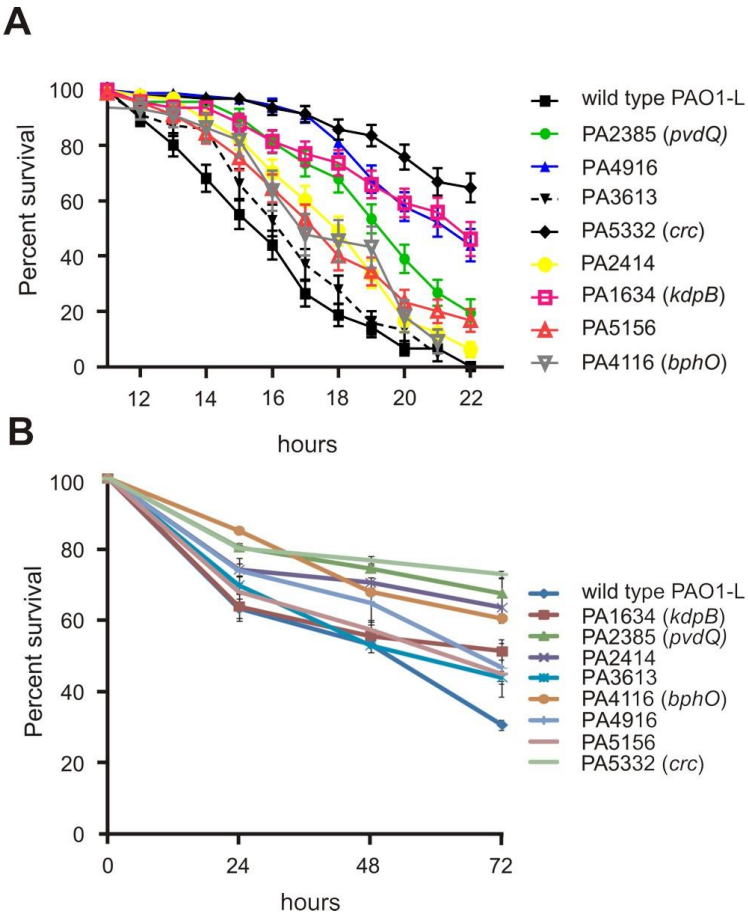
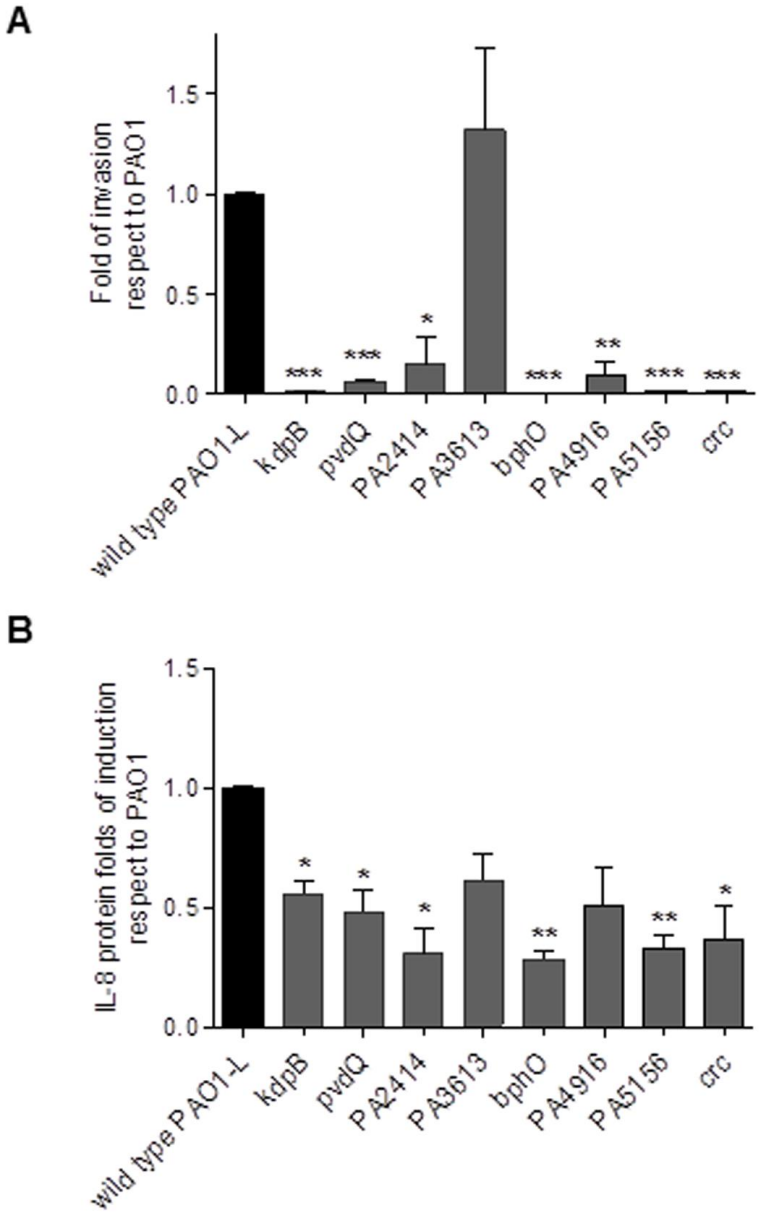


Figure 4


A
Fold of invasion respect to PAO1
1.5
1.0
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wild type PAO1-L
kdpB
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PA2414
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bphO
PA4916
PA5156
crc
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IL-8 protein folds of induction respect to PAO1
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wild type PAO1-L
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PA4916
PA5156
crc

Figure 5

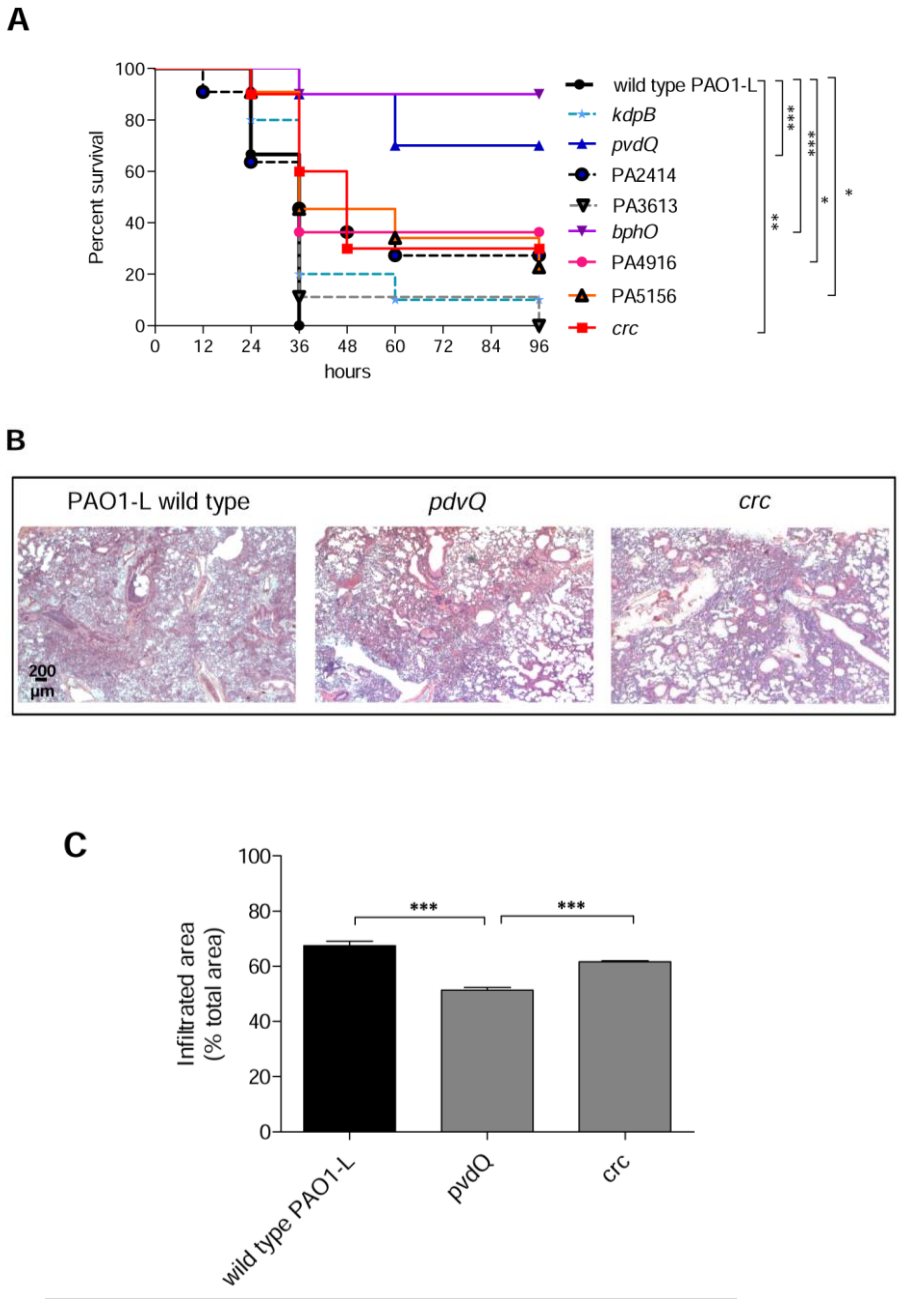

A
Percent survival
hours
wild type PAO1-L
kdpB
pvdQ
PA2414
PA3613
bphO
PA4916
PA5156
crc
B
PAO1-L wild type
pdvQ
crc
200 µm
C
Infiltrated area (% total area)
wild type PAO1-L
pvdQ
crc

Table 1. List of the genes involved in *P. aeruginosa* PAO1-L virulence. The attenuated phenotype is indicated by a red box while not attenuated phenotype is indicated by a blue box.

Locus tag	gene name	Number of Tn5 insertion	class of confidence	Annotated as Virulence Factor[&]	Protease	Swarm	Pyo	C.elegans[~]	D.melanogaster	cytotox A549 pellet[$]	cytotox A549 SN[$]	Homology to P.aeruginosa strains score[*]	Homology to Bcc J2315 score[#]	Homology to E.coli k12 score[#]	Identification in other screenings
PA0082	*tssA1*	2	2	yes								6	1	1	Potvin et al., Environ. Microbiol. (2003) 5, 1294-1308
PA0337	*ptsP*	1	1									6	1	2	Feinbaum et al., PLoS Pathog. (2012) July; 8(7): e1002813
PA0366		1	3									6	2	1	
PA0396	*pilU*	1	1	yes								6	1	1	
PA0425	*mexA*	1	1									6	3	2	
PA0534	*pauB1*	1	2									6	3	1	
PA0593	*pdxA*	1	2									6	2	3	
PA0823		1	4									3	1	1	
PA0844	*plcH*	1	1	yes								6	2	1	
PA0914		1	4									5	0	2	
PA0996	*pqsA*	1	1									6	1	1	
PA0998	*pqsC*	1	1									6	1	2	
PA1030-P8	*P8*	2	4-1												
PA1118		1	4									6	0	2	
PA1523	*xdhB*	1	2									6	2	1	
PA1542		1	4									6	2	1	
PA1634	*kdpB*	7	2									6	2	3	
PA1799	*parR*	2	1									6	2	2	
PA2130	*cupA3*	2	1									5	1	1	Winstanley et al., Genome Res. (2009) Jan;19(1):12-23
PA2240	*pslJ*	1	1									6	0	1	
PA2385	*pvdQ*	2	1	yes								6	0	1	
PA2414		1	2									6	1	2	
PA2781		1	4									6	1	1	
PA2854		2	4									6	UH	1	

PA2998	*nqrB*	1	2									6	0	1	Potvin et al., Environ. Microbiol. (2003) 5, 1294-1308; Bianconi et al., PLoS Pathog. (2011) Feb 3;7(2):e1001270
PA3058	*pelG*	1	1									6	0	1	
PA3071		1	4									6	1	1	
PA3101	*xcpT*	3	1									6	2	2	
PA3110		1	4									5	UH	1	Potvin et al., Environ. Microbiol. (2003) 5, 1294-1308
PA3139		1	3									6	3	2	
PA3257	*prc*	1	2									6	1	1	
PA3270		1	4									6	1	1	
PA3327		1	3									5	1	1	
PA3448		1	3									6	3	2	
PA3449		1	4									6	3	1	
PA3460		3	3									6	0	2	
PA3493		1	4									6	0	2	
PA3613		1	4									6	0	2	
PA3622	*rpoS*	11	1									5	2	3	
PA3649	*mucP*	3	1	yes								6	2	2	
PA3761	*nagE*	1	1									6	2	2	
PA3799		7	4									6	2	2	
PA3867		2	3									6	0	1	
PA3950		1	3									3	3	2	
PA4000		1	4									6	0	1	
PA4059		1	4									6	UH	3	
PA4098		1	3									6	3	1	Winstanley et al., Genome Res. (2009) Jan;19(1):12-23
PA4113		1	3									6	1	2	
PA4116	*bphO*	1	1									6	UH	2	
PA4265	*tufA*	1	2									4	4	4	
PA4282	*sbcC*	2	3									6	0	1	
PA4352		2	4									6	UH	1	
PA4448	*hisD*	1	2									6	3	2	
PA4489		1	4									6	UH	2	Potvin et al., Environ. Microbiol. (2003) 5, 1294-1308
PA4498		1	3									6	1	1	
PA4515		3	4									6	2	2	
PA4528	*PilD*	1	1	yes								6	2	2	Potvin et al., Environ. Microbiol. (2003) 5, 1294-1308
PA4684		1	4									6	0	1	

PA4767-68		2	4-2												
PA4768	*smpB*	1	2									6	2	2	
PA4809	*fdhE*	1	2									6	1	1	
PA4916		1	4									6	0	1	
PA5022		1	4									6	0	1	
PA5138		1	4									6	0	2	
PA5156		1	4									6	1	1	
PA5202-03		1	4-2												
PA5203	*gshA*	4	2									6	2	2	Feinbaum et al., PLoS Pathog. (2012) July; 8(7): e1002813
PA5332	*crc*	1	1									6	1	1	
PA5360-61	*phoB-phoR*	1	1-2												
PA5361	*phoR*	1	2									6	2	1	
PA5548		1	3									5	1	1	

[&] Present in the Virulence Factor Database (http://www.mgc.ac.cn/VFs/)
[*] Number of genome in which the target is conserved, homology >80%
[#] UH uncertain homology
0 homology <20%
1 homology between 20-40%
2 homology between 41-60%
3 homology between 61-80%
4 homology >80%
[~] Infection assays were carried out as described in the Material and Methods. Mutant strains were considered as attenuated when more than 40% of the flies survived 24h post infection
[$] Cytoxicity lower than 4% was defined as highly decreased cytotoxic capacity

Supplementary information

Supplementary material and method

Pyocyanin and pyoverdine production

In order to test pyocyanin production, *P. aeruginosa* strains were grown at 37°C in LB for 16h. The concentrations of pyocyanin were determined in 5 ml culture supernatants as described previously (Essar *et al*., 1990). For pyoverdine production, *P. aeruginosa* cells were grown in CAA medium for 24h (Ongena *et al*., 2002). Pyoverdine production was assessed by determination of the optical density of culture supernatants at 400 nm.

Protease and elastase activity

Protease and elastase activities were assessed by using a colorimetric method (Ohman *et al*., 1980). Briefly, overnight cultures of 100 µl were added to a buffer containing 100 mM Tris, 1 mM $CaCl_2$ (pH 7.5) and 5 mg. ml^{-1} of the substrates azocasein (Sigma) for protease detection assay or elastin congo red (Sigma) for elastase detection assay. The enzymatic reactions mixture were incubated at 37°C, and stopped after 15 min using 500 µl of trichloroacetic acid 10% (v/v) for protease and after 2 h using 100 µl of 120 mM EDTA

for elastase. The level of enzymatic activity was determined by reading the optical density of clear supernatant at 400 mM for protease and 495 mM for elastase.

Motility assays

Twitching

Twitching assay was done using Difco LB broth solidified with 1% (w/v) Difco agar. Twitch plates were briefly dried and strains were stab inoculated with a sharp toothpick to the bottom of the Petri dish from an overnight-grown LB agar (1.5%, w/v) plate. After incubation at 37°C for 24 h, the zone of motility at the agar/Petri dish interface was measured.

Swarming

For swarming motility assay, a volume of 5 µl of an overnight (16 h) bacterial culture was spotted on swarming agar plate containing 5 g. l^{-1} Bacto agar (Difco), 8 g. l^{-1} Nutrient broth N^o2 (Oxoid), and 0.5% (w/v) glucose (Sigma), and incubated at 37^{o}C for 16 h.

Swimming

Media used for swimming assay was Nutrient broth N^o2 (Oxoid), 0.5% (w/v) glucose (Sigma) and 0.3% (w/v) agarose (GIBCO/BRL). Swim plates were inoculated with bacteria from an overnight culture in LB agar (1.5%,

w/v) plates at 37°C with a sterile toothpick. The plates were then sealed with parafilm (3M) to prevent dehydration and incubated at 25°C for 12h.

Biofilm formation

The ability of *P. aeruginosa* PAO1-L to form biofilm was analysed using a bioflux system (Fluxion). Biofilm was grown continuously in microfluidic channels. *P. aeruginosa* PAO1-L wild type and its transposon mutants derivatives tagged with *Tn*7-*egfp* were grown at 37^{o}C for 16h in 2 ml Müller Hinton (MH) (Oxoid) broth. The culture was refreshed, grown for a further 4h before setting up the biofilm experiment, and diluted to OD 0.05 in 7% MH broth. The biofilm was allowed to form in 10% MH broth at a flow rate of 63µl/h (corresponding to a share rate of 2 dyn/cm^2) at a temperature of 37^{o}C for 15h. The microfluidic channel was assayed under a Laser Scanning fluorescent Microscope (LSM2, Zeiss). Biofilm was visualised using light microscope and *egfp* mode at an excitation wavelength of 488nm. Imaging was carried out using Zen 2009 imaging software (Zeiss). A number of 5 replicate images were taken randomly along the microfluidic channel with a Z-stack of 75 images. Biofilm images were analysed for biomass using Comstat 2 statistical software (Heydorn et al., 2000). An automatic thresholding was applied using Ortsus's method.

Supplementary figures

Figure 1S. Phenotypic characterisation of *P. aeruginosa* PAO1-L and its transposon mutants PA1634 (*kdpB*), PA2414, PA2385 (*pvdQ*), PA3613, PA4116 (*bphO*), PA4916, PA5156, PA5332 (*crc*). A) Protease, elastase, pyocyanin and pyoverdine production was determined in supernatant from culture grown to stationary phase using colorimetric assays. For twitching motility cells were inoculated with a toothpick from a LB agar plate onto a twitching plate (tryptone broth plus 1% agar). The diameter of the twitching zone was determined. Values of triplicate cultures are given. **B**) Biofilm formation was assessed using a bioflux microfluidic channel. Biofilm cells were continuously grown in 10% LB at 37°C for a period of 14h. Standard deviations are based on the mean values of six images taken in random locations in the microfluidic channel. **C**) For swarming and swimming motility, cell wer inoculated from a 16h LB culture onto swarming or swimming plates containing 0.5% or 0.3% agar, respectively.

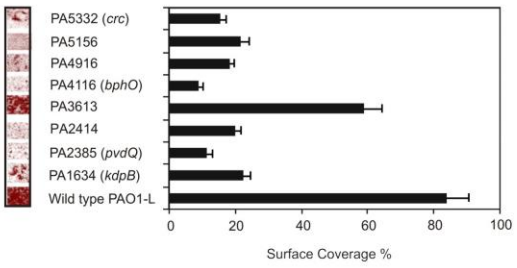
Figure 2S: Survival percent at different infection doses of wild type PAO1-L in C57Bl/6NCrl. C57Bl/6NCrl mice were intratracheally infected with escalating doses of *wild type* PAO1-L ranging from $5x10^5$ to 10^7 CFU. Survival of infected mice was followed over a period of 4 days and is indicated as percent. The first lethal dose was $5x10^6$ CFU. Two independent experiments were pooled. * $p<0.05$; ** $p<0.01$ (Mantel-Cox test).

Figure 1S

A

Bacterial strain	Elastase (OD_{495} /OD_{600})			Pyoverdine (OD_{400} /OD_{600})	Twitching (cm)
	4h	8h	16h		
Wild Type PAO1-L	0.04 ± 0.01	0.06 ± 0.01	0.93 ± 0.02	6.73 ± 0.07	1.5 ± 0.01
PA1634 (*kdpB*)	0.01 ± 0.00	0.03 ± 0.00	0.42 ± 0.01	5.49 ± 0.13	1.3 ± 0.01
PA2385 (*pvdQ*)	0.01 ± 0.00	0.04 ± 0.00	0.61 ± 0.01	0.12 ± 0.09	1.0 ± 0.02
PA2414	0.02 ± 0.00	0.02 ± 0.00	0.46 ± 0.01	4.11 ± 0.12	1.3 ± 0.01
PA3613	0.04 ± 0.01	0.05 ± 0.01	0.75 ± 0.02	6.92 ± 0.08	1.5 ± 0.01
PA4116 (*bphO*)	0.02 ± 0.00	0.02 ± 0.00	0.42 ± 0.01	3.18 ± 0.07	1.8 ± 0.02
PA4916	0.02 ± 0.00	0.02 ± 0.00	0.35 ± 0.00	5.34 ± 0.14	0.0 ± 0.00
PA5156	0.02 ± 0.00	0.04 ± 0.00	0.57 ± 0.01	6.59 ± 0.09	0.0 ± 0.00
PA5332 (*crc*)	0.01 ± 0.00	0.02 ± 0.00	0.21 ± 0.01	3.47 ± 0.11	0.0 ± 0.00

B



C

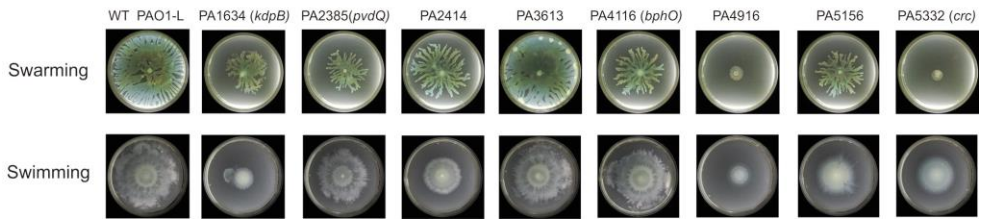
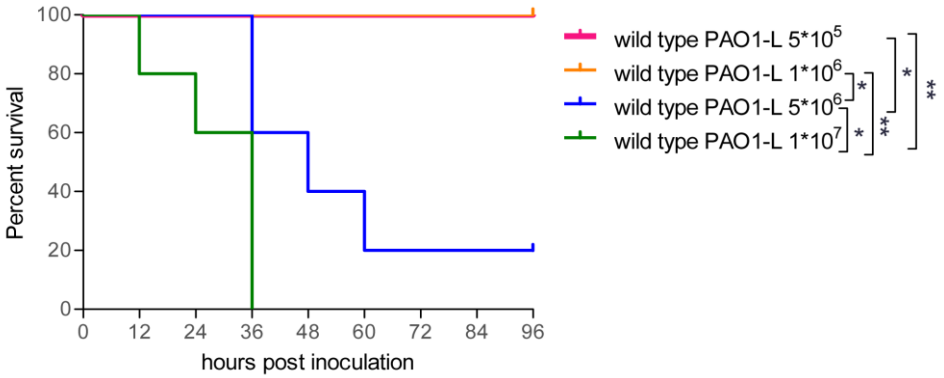


Figure 2S



Supplementary table

TABLE 1S. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Pseudomonas		
PAO1-L	PAO1 Lausanne wild type	Lausanne collection
Escherichia coli		
λ*pir* S17.1	*Thi proA hsdR recA RP4-2-tet*::Mu-1 *kan*::Tn*7*integrant (Tp^r Sm^r)	Metcalf et al. (1994)
Plasmids		
pRL27	Plasmid harbouring Tn5 transposon, Km^r	Larsen et al. (2002)
pLM1	Plasmid harbouring Tn5 transposon derived from pRL27, Gent^r	Larsen et al. (2002)

TABLE 2S. Percentage of homology of the amino acid sequence of the selected targets respect to *P. aeruginosa* strains LESB58, PA14, PA7, 2192, C3719 and

Locus tag	gene name	% ID								
		LESB58	PA14	PA7	2192	C3719	PACS2	Bcc J2315	*Ec* K12	Human homologs
PA0082	*tssA1*	98	98	90	99	98	97	27	29.7	0
PA0337	*ptsP*	100	100	99	100	100	100	35	42	0
PA0366		100	99	98	100	100	100	51	29	0
PA0396	*pilU*	100	99	99	99	100	100	33	37	0
PA0425	*mexA*	100	100	99	100	99	100	70	55	0
PA0534		100	99	96	100	99	99	67	40	0
PA0593	*pdxA*	99	99	95	99	99	99	54	63	0
PA0823		98	98	30	37	32	98	31	34	0
PA0844	*plcH*	99	99	96	99	99	99	42	32	0
PA0914		99	99	78	99	99	98	UH	44	0
PA0996	*pqsA*	100	99	86	100	100	100	29	28.3	17
PA0998	*pqsC*	100	100	97	100	100	100	27	50	0
PA1030/P8	*P8*									
PA1118		99	97	86	98	100	98	0	41	0
PA1523	*xdhB*	99	99	98	99	99	99	55	25	2
PA1542		100	100	95	100	100	100	44	27	0
PA1634	*kdpB*	99	99	98	99	100	99	60	60.6	23
PA1799	*parR*	100	99	94	100	100	100	50	42	0
PA2130	*cupA3*	99	99	69	99	98	99	27	31	0
PA2240	*pslJ*	100	100	99	100	100	100	UH	26	0
PA2385	*pvdQ*	99	99	94	99	99	99	UH	32	0
PA2414		99	99	94	99	99	99	25	41.4	0
PA2781		99	99	97	92	99	99	29	39	0
PA2854		100	100	98	100	100	100	UH	40	0
PA2873		99	99	88	98	98	98	UH	48.7	0
PA2944	*cobN*	100	99	96	99	UH	99	57	23	0
PA2998	*nqrB*	100	100	99	100	100	100	UH	29.1	0
PA3058	*pelG*	100	99	96	100	100	99	UH	29	0
PA3071		100	99	96	100	100	100	32	38	0
PA3101	*xcpT*	99	98	99	99	99	99	63	55	0
PA3110		99	96	94	64	99	100	UH	26.1	0
PA3139		100	99	97	100	100	100	73	52	3
PA3257	*prc*	100	100	99	100	100	100	31	36	0
PA3270		99	100	95	100	100	100	37	30	0
PA3327	*NRPS*	99	99	34	99	99	99	32	35	0
PA3445		100	99	99	100	99	100	65	48	0
PA3448		99	99	98	100	100	100	66	43.1	0

PA3449		99	99	97	99	99	99	63	31	0
PA3460		100	100	98	100	99	100	UH	44	0
PA3493		100	100	86	100	94	100	0	46	0
PA3613		99	99	97	100	100	100	0	43	0
PA3622	*rpoS*	100	100	99	100	UH	100	46	76	0
PA3649	*mucP*	99	99	97	99	100	99	41	46	0
PA3761	*nagE*	99	99	94	99	99	99	55	47	0
PA3799		99	99	100	100	100	100	55	59	1
PA3867		39	98	96	97	40	36	UH	36	0
PA3950		100	99	95	94	99	99	70	51	42
PA4000		99	99	97	99	89	99	UH	38.7	0
PA4059		99	98	96	98	97	98	UH	62	1
PA4098		100	100	96	99	100	100	63	34	0
PA4113		99	99	95	99	99	99	31	58	0
PA4116	*bphO*	99	99	85	98	100	100	UH	42	0
PA4130		99	99	97	99	99	99	66	32.2	0
PA4265	*tufA*	100	100	100	31	31	100	82	84	17
PA4282	*shsC*	99	98	81	98	99	99	UH	28	0
PA4352	*usp*	100	100	98	100	100	100	UH	22	0
PA4448	*hisD*	100	100	98	100	99	100	69	41	0
PA4489		99	99	98	99	99	99	UH	53.3	1
PA4498		99	99	90	86	99	99	33	34	0
PA4515	*piuC*	99	99	96	99	99	99	58	49	0
PA4528	*PilD*	99	98	95	99	99	99	49	41.2	0
PA4684		99	100	99	99	99	100	UH	36	0
PA4767-68										
PA4768	*smpB*	100	100	100	100	100	100	58	59	0
PA4803		100	100	33	100	100	100	UH	24.3	1
PA4809	*fdhE*	99	99	98	99	99	99	38	39	0
PA4916		99	100	98	99	99	99	0	31	0
PA5022	*aefA*	100	99	96	99	100	99	0	39	0
PA5132		98	98	96	98	92	99	38	27	0
PA5138		100	100	98	100	100	100	0	56	0
PA5156		99	99	94	99	98	99	26	37	0
PA5202-03										
PA5203	*gshA*	99	99	96	99	99	99	53	42	0
PA5332	*crc*	100	100	99	100	100	100	40	32.6	1
PA5360-61	*phoB-phoR*									
PA5361	*phoR*	99	99	99	99	100	100	41	40	0
PA5548		99	98	28	99	99	98	31	23	0

PACS2 and respect to *B. cenocepacia* J2315, *E. coli* K12 and humans.

UH uncertain homology