Insights into host-pathogen interactions from state of the art animal models of respiratory *Pseudomonas aeruginosa* infections

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Abstract:

Pseudomonas aeruginosa is an important opportunistic pathogen that can cause acute respiratory infections in immune competent patients or chronic infections in patients with cystic fibrosis or other immunocompromised individuals. When acquiring the chronic infection state, bacteria are encapsulated within biofilm structures enabling them to withstand diverse environmental assaults, including immune reactions and antimicrobial therapy. Understanding molecular interactions within the bacteria as well as with the host or other bacteria is essential to develop innovative treatment strategies. Such knowledge might be accumulated *in vitro*. However, it is ultimately necessary to confirm these findings *in vivo*. In the present review, we have described state of the art *in vivo* models that allow studying *P. aeruginosa* infections in molecular details. The portrayed mammalian models exclusively focus on respiratory infections. The data obtained by alternative animal models which lack lung tissue, often provide molecular insights that are easily transferable to the mammalian situation. Importantly, all these surrogate *in vivo* systems reveal complex molecular interactions of *P. aeruginosa* with the host. A critical assessment of the advantages and disadvantages of such models is provided.

Introduction

Pseudomonas aeruginosa is a clinically highly relevant opportunistic pathogen that is known to cause devastating acute as well as chronic persistent infections. Ithas a comparatively large genome size harboring a multitude of genes providing fitness and survival advantages invery heterogeneousenvironments. *P. aeruginosa*exhibits the capacity to quickly adapt gene expression

profilesto changing environmental conditions [1,2]. Thus, *P. aeruginosa* can be found ubiquitously not only in the environment but also in nosocomial infection settings.

As a pathogen, *P. aeruginosa* entertains the expression of an impressive repertoire of virulence factors such as proteases, type 3 secretion system effector molecules, siderophores as well as rhamnolipid surfactants and phenanzines, among others [3–5]. Quorum sensing allows *P. aeruginosa* to orchestrate the expression of those virulence factors in a cell-density dependent manner [6].The bacteria arefurthermore able to efficiently regulate the switch from a highly virulent and motile single cell mode of growth to a protected sessile multicellular biofilm lifestyle. In such biofilms, the bacteria are encapsulated in a self-produced extracellular matrix, which consists of different components including the polysaccharide alginate, and polysaccharides like Psl/Pel as well as proteins and nucleic acids [7]. The nature of this matrix successfully protects the bacteria from harsh surrounding environments *e.g.* the immune system of the host and/or antibiotics. This biofilm shield further more allows the bacteria to develop acquired and adaptive antibiotic resistances [8]. Consequently applied antibiotic treatments become less effective and eventually ineffective.
Biofilm-associated chronic *P. aeruginosa* infections are prevalently observed in patients who suffer from severe burn wounds, diabetic feet, infections of the ear (otitis), the cornea (keratitis) and the lung especially in cystic fibrosis (CF) patients.

The biofilm shield allows the bacteria to develop acquired and adaptive antibiotic resistances [8]. Consequently, antibiotic treatments become less effective and eventually ineffective. In general, *P. aeruginosa* is naturally highly resistant against antibiotics. Its outer membrane permeability is approx. 100-fold lower than that of other Gram-negative bacteria [9]. This serves as a significant barrier for the penetration of antibiotics such as beta-lactams and quinolones, which leads to intrinsic resistance to the antibiotics. Additionally low permeability of antibiotics synergizes with other resistance mechanism such as activation of efflux pumps. Similarly, antibiotic exposure also results into selection of a large array of chromosomal gene mutations to acquire resistance, against many clinically relevant antibiotics [8]. In the biofilm mode, P. *aeruginosa* also uses intrinsic mechanisms to gain antibiotic resistance but commonly also uses horizontal gene transfer strategy acquire resistance from different bacterial species [10][11].

Bacterial pathogens alert the defense systems of the host mainly via their so-called pathogen-associated-molecular-pattern (PAMP). PAMPs are molecular structures in which microorganisms can be differentiated from the host. For instance, typical bacterial PAMPs are lipopolysaccharides (cell wall component of gram-negative bacteria), lipoteichoic acid (cell wall component of gram-positive bacteria), glycol- and lipoproteins, flagellin and nucleic acids (containing for instance unmethylated CpG motifs)[12,13]. These conserved patterns bind to so-called pattern-recognition-receptors (PRRs) of the host which can be found on the surface of host cells but also intracytosolic. Interestingly, the receptors and the corresponding signaling cascades found in invertebrates are highly homologous to receptors and cascades found in the innate immune system of vertebrates. For instance, the two signaling cascades of Drosophila that are activated by either fungi and gram-positive bacteria or gram-negative bacteria end in the activation and nuclear transport of the transcription factors DIF or relish, respectively [14]. Both transcription factors are homologs to transcription factors belonging to the NFκB family that is found in vertebrates and represent the major transcription factors regulating inflammatory and immune reactions [15].

The defense effectors of invertebrates are almost exclusively restricted to an innate type of immune system. They consist of humoral effectors like anti-microbial peptides, lectins and complement like molecules or cellular mechanisms that include phagocytosis, encapsulation and production of reactive oxygen (ROS) and reactive nitrogen species (like NO). These mechanisms are similarly found in vertebrates. However, in such animals additionally highly specific mechanisms have developed, like antibodies allowing antibody dependent complement activation and opsonisation of microorganisms for phagocytosis or T cells that specifically activate other cells to fight against the microorganism. Above all, such specific responses include an immunological memory that enables a strongly enhanced response when the individual is invaded by the same pathogen again.

Presently, most of the knowledge on physiology of *P. aeruginosa* is derived from bacterial cultures for which an excessive *in vitro* tool box has been established. However, such data need to be complemented by insights from *in vivo* models. To date, a variety of such animal systems have been introduced, which range from invertebrate to mammalian models. Consequently, in this context recently several general and specific reviews have comprehensively summarized animal infection models for *P. aeruginosa* (*Caenorhabditis elegans*: [16–18];insects: [19–21];general invertebrate models: [22]; vertebrate models: [23–26]).

Here, we restrict ourselves to highlighting some recent developments to study *P. aeruginosa* pathogenicity *in vivo*. For instance, recently Ha *et al*. and Bjarnsholt *et al*. reviewed studies on *P. aeruginosa* biofilms, which give clear insights into development and treatment strategies for *P. aeruginosa* biofilm infections [27,28]. In addition, we survey a few latest findings on *in vivo* testing of new compounds or strategies to fight *P. aeruginosa*. We regret that we had to limit our choice of publications and humbly apologize for omitting others.

Models for *in vivo* infection by *P. aeruginosa*

1. Invertebrate models

Invertebrate infection models have certain advantages. They are comparatively easy to establish and to maintain. They are cost effective and do not require extensive ethical consideration as is necessary for vertebrate models. Although they lack an adaptive immune system[29], invertebrates are capable of reacting strongly towards infectious agents. Thus, invertebrate infection models could be considered simplified models for innate immunity. The following paragraphs summarize the three main invertebrate infection models for *P. aeruginosa*

1.1. Nematodes

Caenorhabditis elegans is a soil resident nematode. Due to its unproblematic housing under laboratory conditions, comparable simple body organization and hermaphroditic lifestyle ,*C. elegans* became one of the favorite study objects of cell biologists and geneticists [30]. Infection studies using *C. elegans* can profit from a host-manipulated background in genetically well-characterized organisms [31,32]. As the nematode is feeding mainly on bacteria, ingestion of pathogenic bacteria like *P. aeruginosa* may be encountered. Thus *C. elegans* developed defense mechanisms. The organism consists only of 959 cells. It does not possess any lymphoid organs or professional immune cells. However, it can release C-type lectin containing proteins, lysozymes, antimicrobial peptides and

express CUB(for complement C1r/C1s, Uegf, Bmp1) domain proteins that are involved in multiple functions including inflammation and tissue repair [33]

Pyocyanin is a secondary metabolite and an important virulence factor of *P. aeruginosa.* It has been extensively studied in the *C. elegans* system. It is secreted into the environment and plays a crucial role in establishing infections. Elevated pyocyanin levels were shown to positively correlate with clinical pulmonary exacerbations [34]. Already one and a half decades ago the group of Tan *et al.* [35]could show, that the influence of pyocyanin on the host can be studied in nematodes. This has led to the discovery of additional phenanzines that exhibit toxic effects on the host in a partially pH dependent manner [36]. Especially phenancine-1-carboxilic acid could be shown to be toxic under acidified conditions as caused by *P. aeruginosa* infections. Such effects might also be relevant for toxicity of *P. aeruginosa* in mammals and plants. Interestingly, a *P. aeruginosa* mutant exhibiting a defective intracellular multiplication factor *icmF* gene, encoding for parts of the type VI secretion system, showed an increased pyocyanin production but was found to be nonvirulent in *C. elegans* [37]. However, this mutation shows additional pleiotropic effects like interference with the quorum sensing control repressor QscR, possibly reconciling this controversy.

Infected *C. elegans* respond to pyocyanin with mitophagy, the lysosomal degradation of damaged mitochondria [38].Via this mechanism, the organisms are able to survive and withstand the infection. Autophagy was also noticed as a defense mechanism of *C. elegans* against *P. aeruginosa*. Apparently, the bacterial killing is not directly associated with autophagy, but it could be shown to be necessary to remove necrotic cells from the infected intestine that are caused by the bacterial infection. Autophagy is induced by the bacteria in the gut resulting in upregulation of the EGF homologLIN3. This leads to the activation of the MAPkinase ERK cascade via the EFGR homolog LET2. In the absence of this mechanism, the intestine can be easily breached by the bacteria and the infection becomes systemic. A similar scenario can be envisaged in mammals or humans in chronic inflammatory bowel disease [39]. This clearly demonstrates the value of employing *C. elegans* as infection model.

Importantly, the overall resource-saving working with the *C. elegans* model system allows also the initiation of global screenings for candidates of inhibitory compounds [40,41]. Similarly, the nematodes provide a simple system to test the fitness of newly derived antibiotic resistant *P. aeruginosa* [42]. Thus, *C. elegans* can be considered a bridge between *in vitro* systems (*e.g.* cell culture) and the most complex vertebrate *in vivo* models. This takes the evaluation of new compounds a step further on the way to the establishment of translational potential.

The potential of this model system can also be seen from experiments were metabolomics was applied to infected *C. elegans*. Here, an improved high throughput in-depth non-targeted metabolomics analysis revealed specific metabolic phenotypes of *C. elegans* upon infection. The obtained metabolite profiles showed differences in amino acid and sugar metabolism between *P. aeruginosa* and *Salmonella enterica* infections. Strikingly this approach even allowed the discrimination between a *P. aeru*ginosa *gaga* mutant and its parental strain by changes in energy metabolism[43].

Although the *C. elegans* models proved to be a powerful tool for infection research, there are drawbacks. First of all, the uptake of the bacterial pathogens cannot be influenced as the worms are feeding on *P. aeruginosa* and only a limited number of bacteria can infect a single worm. Thus, for instance, experiments using dense transposon mutant libraries for efficient genome-wide screening for infection relevant genes might face the problem of a serious bottleneck. Additionally, *C. elegans*

has no professional immune cells and is apparently not able to signal via the TLR/NF-κB pathway - a highly conserved pathway of cell signaling found in the animal as well as in the plant kingdom[44], This may limit the informative value as a model for human conditions.

Nevertheless, the described examples clearly illustrate the broad potential of the *C. elegans* model for research on *P. aeruginosa*. Single virulence factor testing, inhibitory compound evaluation and the host immune response studies are only some possible model applications. They all benefit from the hermaphroditism of *C. elegans*, supplying researchers with large populations of genetically identical animals, thus scaling down the influence of individual variation.

1.2. Insects

Insects such as *Galleria mellonella* or *Drosophila melanogaster* exhibit certain defense mechanisms and structures, which are not present in the nematode. This favors them for the use as invertebrate infection models. The cellular response of insects against bacterial infection is usually characterized by phagocytosis, nodulation and encapsulation of the pathogen. In addition, the humoral response of insects comprises coagulation, melanisation and especially the induction of antimicrobial peptides. The insect immune compartments are the fatbody (liver equivalent), epithelial and mucosal surfaces and the hemolymph (insect blood) with certain innate immune cells. For example, hemocytes can act as professional phagocytes, like mammalian neutrophils or macrophages. They are able to take up bacteria and destroy them (for reviews on the insect immune system see [45,46]).

Galleria mellonella, the greater wax moth is an organism belonging to the order of lepidoptera. It is further known as the honeycomb moth, because female wax moths lay their eggs in beehives, where the larvae hatch and feet on the wax and pollen available in the honey comb. The larvae of this moth have a size ranging from 12-20 mm. They have proven to be worthy model organisms and are increasingly used for bacteria-associated infection research. Wax moth larvae can be incubated at 37 °C, and thus at the natural mammalian host temperature. Due to the size of the caterpillar direct injection of small volumes of bacterial cultures can be easily achieved. Alternatively, force feeding can be used. The caterpillars can usually be bought from animal food stores and thus do not need any special breeding and maintenance provisions in the lab. Last but not least, a fatal infection can be easily monitored by melanisation of the caterpillar (for detailed infection protocols, readers are referred to [21]).

The value of the Galleria larvae model with regard to testing virulence factors of *P. aeruginosa* that also affect mammals was systematically assessed already 15 years ago [47]. Thirty-two mutants in virulence-associated genes were compared in the Galleria and the mouse model. A clear positive correlation between the two models was observed for all such genes. This proofs the value of wax moth caterpillars as simple convenient *in vivo* model being as close to mammals as an invertebrate model might get.

Especially for prescreening the efficacy of antibiotic components, the Galleria *in vivo* system has gained popularity lately. For instance, the combinatorial potential of dual or triple antibiotic therapy against a multi-drug-resistant *P. aeruginosa* strain has revealed novel strategies of treatment [48]. Similarly, the pharmacokinetics of known antibiotics against *P. aeruginosa* has been carried out in this system [49].Moreover, the optimization of the antibiotic delivery strategies via nano particles [50] or the treatment *P. aeruginosa* by antibiotics combined with efflux pump inhibitorshas been

evaluated [51]. Finally, strategies to counteract virulence factors like pyoverdine [52] or quorum sensing effectors [53]were explored in the Galleria model.

Drosophila melanogaster, the fruit fly is a favored subject for genetic and developmental research. Although, the potential of *D. melanogaster* as a model organism is still far from being fully explored, the small insect was shown to be of use for bacterial infection studies. Being approximately 2.5 fold bigger than *C. elegans, D. melanogaster* offers the possibility to target specific organs for infection, like the thorax, hemolymph or intestine. There are two major infection models: i) the feeding modelin which the flies take up the pathogen via the intestine. This allows studying the pathogenesis for several days, ii) the septic injury model in which the animals are pricked with needles carrying the bacteria. Pathogenesis can be monitored for a few days only. However, the infection of *D. melanogaster* is not limited to acute infections. *P. aeruginosa* is capable to form microcolonies, possibly resembling biofilms, in the crop and intestine of infected flies (reviewed by [54]).

The power of Drosophila as infection model for *P. aeruginosa* has been impressively shown by co-infection experiments using quorum sensing mutants. In *P. aeruginosa* two quorum sensing regulatory systems exist – *lasIR* and *rhlIR.* The LasIR system depends on N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) as effector molecule and RhlIR on N-butanoyl-L-homoserine lactone (C4-HSL), respectively [55]. Although the two quorum sensing systems orchestrate the cell-density dependent production of important virulence factors, the activity of these systems also seems to represent a serious burden for the bacteria. Inactivating mutants are often found in clinical isolates recovered from chronic infections [56–58]. These mutants may be so-called cheaters that do not produce but utilize relevant compounds provided by cooperating partners [59,60]. Lutter *et al.* compared isolates exhibiting such mutations with wild-type (Wt) *P. aeruginosa.* While in the nicking system the mutants behaved almost like the Wt, in the feeding system all the mutants were avirulent demonstrating that the presence of both quorum sensing system is required to infect the flies. When the mutants were supplemented during the Drosophila infections with extracts of Wt *P. aeruginosa* or purified 3-oxo-C12-HSL or C4-HSL, the mutants became lethal again [61]. This impressively demonstrates the versatility of the Drosophila *in vivo* system. The Wt and the mutants were also tested in co-infection experiments. All such mutants cooperated with the Wt. This was in clear contrast to experiments in the burned mouse model and the wax moth, where quorum sensing mutants usually behave as cheaters [47,62]. It will be interesting to characterize the mechanisms leading to this controversy.

Reactive oxygen species (ROS) like superoxide, hydrogen peroxide and hydroxyl radicals are generated under physiological aerobic conditions by *P. aeruginosa* itself but also by phagocytic and other host cells upon infection. These antimicrobial effectors target a broad spectrum of molecules like bacterial DNA, RNA, proteins and lipids, thus interfering with many aspects of their physiology. Consequently, *P. aeruginosa* has developed many different defense mechanisms to withstand the encounter of such reactive molecules. The employment of the *D. melanogaster* model allowed the easy comparison of several genes involved in the anti-oxidant response. For instance, flavodoxins (Fld) representbacterial mobile electron shuttle proteins with anti-oxidant capacities. Recently a new, long-chain, Flavodoxinof *P. aeruginosa*, FldP, was found and characterized. It represents a virulence factor since it conferred bacterial survival in the fly infection system and was required for causing death of most of the flies [63].

Exposure to ROS results in the oxidation of free and protein bound methionine. Therefore, by oxidative stress the expression of repair enzymes like methionine sulfoxide reductases is induced in *P. aeruginosa*. Accordingly, the methionine sulfoxide reductase genes *msrA* (PA5018) and *msrB* (PA2827) of *P. aeruginosa* are responsible for oxidative stress protection. When these enzymes are nonfunctional, the corresponding mutants were shown to be highly attenuated in the Drosophila model [64]. It could also be shown that the two enzymes act additively because the double mutant exerted a more serious phenotype as compared to the single mutants. In addition, the double mutant could only partially be complemented by either *mrsA* or *mrsB*.

As mentioned, production of ROS is not limited to the host. Streptococci from the oral cavity were observed to produce hydrogen peroxide. Moreover, streptococci can colonize the lungs of CF patients and therefore potentially interact with *P. aeruginosa*. The studies of Scoffield *et al.* [65,66] investigated the polymicrobial interplay of hydrogen peroxide producing streptococci and nitrate reductase deficient *P. aeruginosa*. They could demonstrate that the growth of *nirS* deficient *P. aeruginosa* is inhibited in the presence of hydrogen peroxide and nitrite *in vivo*. Interestingly Drosophila infected with *P. aeruginosa* nirS mutant had the highest survival when co-infected with hydrogen peroxide producing streptococci. Although, the mechanisms underlying this interplay of bacteria as well as the host remains elusive. However, it is able to illustrate how suitable insects and their immune features are for infection-related research.

In summary, all these examples highlight the value of the fruit fly and greater wax moth larvae as infection models. However, the limited infection dose of bacteria per individual insect and especially the lack of an adaptive immune system may require a vertebrate model system for more definite characterizations of molecular interaction between the bacteria and the host.

2. Vertebrate animal models

Human diseases might be studied in invertebrate species to a certain extent. However, in the race of arms between host and pathogen, several pathogenic microorganisms are capable to overcome the barriers of the innate immune system. While in more primitive animals the defeat of the innate immune system leads inevitably to the death of the host, in higher animals an adaptive immune system might efficiently deal with infections under these conditions.

2.1. Zebrafish *Danio rerio*

The adult zebrafish, *Danio rerio*, exhibits a fully developed innate and adaptive immune system. However, a temporal separation exists between the maturation of the two immune systems depending on the developmental stages of the fish. In the first day post fertilization, phagocytic innate immune cells develop - first macrophages then neutrophils. Adaptive immune cells are still absent. Five days after fertilization, immature lymphoblasts can be found, which finally mature into functional B and T cells [67]. Thus, depending on the developmental stage at which the infection is applied, exclusively the innate (larvae) or innate and adaptive systems (adult) is taking part in the Pseudomonas-induced immune response. Importantly, it is possible to inhibit expression of particular genes by injecting so-called morpholino anti-sense RNA at the single cell stage of development. Thus, it is possible to inhibit expression of immune effectors or deplete cells of the immune system in this organism by targeting essential transcription factors. Although the zebrafish is equipped with gills

instead of lungs and consequently shows no direct link to CF-associated disease, the traceability and genetic characterization renders *D. rerio* a valuable *in vivo* model system.

The larvae of the zebrafish are small in size (3-5 mm) and available in large quantities, as females can produce up to 300 eggs per week. The fertilized eggs are transparent and develop rapidly. This allows monitoring the progress of infections at real-time given that appropriate fluorescent markers are employed. Bacteria can be injected intravenously for systemic infection or into cellular compartments like the hindbrain ventricle or the pericardium for a localized infection. This could exemplarily be seen in the work of Rocker *et al.* [68]. They introduced *P. aeruginosa* into the hindbrain ventricle of the larvae thus avoiding systemic dissemination and rapid death of the larvae. This allowed them to study the formation of bacterial microcolonies. Formation of such microcolonies is understood as an early step in biofilm development. This interpretation was in agreement with the fact that a Psl mutant, which is no longer able to produce the Psl exopolysaccharide, was less virulent in this system.

The versatility of the zebrafish as an infection model was most impressively demonstrated by Peterman *et al.* [69]. They were interested in the role of the mitochondrial superoxide dismutase (SOD2) under infectious conditions. Reactive oxygen species (ROS) are produced by the host as a defense reaction but they will attack besides the pathogen also self-components like DNA, proteins or lipids. Thus, to protect itself from ROS, the host expresses anti-oxidant enzymes like superoxide dismutases. SOD2 detoxifies ROS within the mitochondria. By infecting zebrafish with *P. aeruginosa*, it could be shown that SOD2 is upregulated in hematopoietic and myelopoietic organs [69]. By deleting SOD2 and infecting the larvae early in development when only macrophages are present, they could show that ROS needs to be detoxified in such cells. The SOD2 depleted animals were more susceptible to the systemic infection and exhibited increased mortality. Moreover, the lack of SOD2 was also shown to have an impact on the phagocyte population under normal conditions. Low number of macrophages and neutrophils were present in such animals. ROS scavengers reverted these phenotypes. The authors suggest that SOD2, besides its role in inflammatory responses, is required for the homeostasis of hematopoietic stem cells that give rise to phagocytes. They also conclude that during chronic inflammatory reactions, cancer as well as sepsis in humans might benefit from antioxidants targeting ROS. First clinical data support this interpretation.

On the other hand, also the defense mechanisms of the pathogens against ROS can be studied in zebrafish. For instance, the transcriptional regulator PA2206 of *P. aeruginosa*is is induced by ROS-stress. It activates approx. 58 genes and is essential for the successful infection of zebrafish [70].

Moreover, zebrafish model can also be exploited for the characterization of new concepts and alternative treatment strategies for *P. aeruginosa* infections. As promising producers of new potent components, members of polymicrobial communities were investigated. The constant competition for resources might lead to the secretion of promising bioactive factors potentially manipulating, antagonizing and/or eliminating the unwanted microbial neighbors. For example *Burkholderia cenopacia* and *P. aeruginosa* are frequently found co-colonizing the lungs of CF patients. *B. cenopacia* produces Cis-2-dodecenoic acid, a Burkholderia diffusible signal factor (BDSF) which was found to inhibit the biofilm formation of non-Burkholderia species. Interestingly BDSF was also shown to interfere with *P. aeruginosa* signaling by influencing the type 3 secretion system and biofilm formation *in vitro* [71]. Furthermore, the co-injection of *P. aeruginosa*and BDSF into adult zebrafish led to an attenuated virulence of *P. aeruginosa* and reduced fish lethality. Similarly, the

putative anti-pseudomonal activity of EPS273, an exopolysaccharide produced by the marine bacterium *P. stutzeri* was explored. Interestingly EPS273 showed anti-biofilm properties *in vitro*. When *P. aeruginosa* pretreated with EPS273 were fed to zebrafish embryos a significantly reduced lethality rate was observed [72]. Unfortunately, both publications did not address the anti-biofilm activity of their components in their *in vivo* setting.

Despite of all the potential *D. rerio* displays as an infection model, certain drawbacks must be considered. Due to the increasing complexity of the vertebrate model organism ethical issue concerning animal well fare and advanced animal husbandry generally complicate the application of the system. Besides, the housing temperature of *D. rerio* is 28 °C. Thus one important environmental clue is different from the human host.

2.2. Mammalian models to study *P. aeruginosa* infections

A plethora of diverse mammalian species has been employed as model systems to study infectious diseases, ranging from Chinchilla to swine and non-human primates. Such models are specifically suitable to analyze different pathogenic manifestations of *P. aeruginosa* infections, including biofilm-associated infections. A recent review provides the interested reader with extensive overview tables of *in vivo* models for the studies of chronic wound infections, chronic otitis media, device-related infections and subcutaneous infections, among others [73]. In the present review we focus exclusively on recent work on *P. aeruginosa* associated respiratory infections.

Acute respiratory infections – The study of non-CF related pneumonia

The pathogenic potential of *P. aeruginosa* and the relevance of single virulence factors for the establishment and maintenance of infectious diseases can be effectively tested in acute lung infection models, which provide a complex multifactorial background. The inoculation of *P. aeruginosa* into the murine respiratory tract forces the bacteria not only to withstand nutritional limitations, the spatial peculiarities of the lung and the host defense system, but they can also be challenged with additional threats like antibiotics. Most of the information on *P. aeruginosa* infections has been obtained using small rodent models, mainly the mouse. Upon bacterial administration either intranasal, intratracheal or oropharyngeal, acute *P. aeruginosa* lung infections with strong inflammatory responses are elicited. Whereas, the route of bacterial administration only marginally affects the final colonization of the lung [74,75], the infection dose, the bacterial strain and the genetic background of the host strongly influences the infectious outcome. This can vary from rapid bacterial clearance to severe sepsis and death [76,77].

A systematic survey on different mouse strains revealed significant differences among the strains tested and the immune response elicited [78],[79]. Mapping and characterization of genetic differences will eventually lead to a deeper understanding of the molecular interaction of the pathogen with its host in much more detail.

Contribution of bacterial factors to pathogenesis. The acute murine lung infection model is often used to confirm the role of bacterial factors that had been extensively characterized *in vitro*. For instance, a recent study focused on the hemolytic phospholipase C (PlcH) that is involved in the degradation of phospatidylcholine a constituent of the lung surfactant [80]. It could be shown that

metabolites like choline regulate ANR dependent promoters that are induced under low oxygen conditions. Interestingly, *plcH* and its regulator *gbdR* were involved in biofilm formation on lung epithelial cells as well as in colonization of murine airways in an acute lung infection system. Since genes of the ANR regulon are also highly expressed in *P. aeruginosa* isolated from sputum of CF patients, PlcH, GbdR or ANR might represent targets for the development of alternative treatment strategies.

Additionally, the importance of metabolic fatty acid pathways on *in vivo* fitness was further shown by Zarzycki-Siek *et al.* [81]. After transport into the bacteria, fatty acids have to be esterified to coenzyme A before they can undergo oxidative degradation. This is achieved by fatty acid-CoA synthetases of which two homologues have been described (FadD1/D2). However, *P. aeruginosa* seems to express 4 additional genes of the FadD family. In an *in vivo* lung infection system, the sextuple *fadD* mutant showed reduced fitness 24 h post infection, which was significantly less than that of the fadD1/D2 double mutant. This indicates a non-redundant role of such fatty acid-CoA synthetases during mammalian infection.

Similarly, the *P. aeruginosa* gene encoding SphA (PA5325) was shown to be involved in bacterial pathogenesis as deletion of *spar* or *sphA* led to reduced colonization of the murine lung. Apparently, this gene is induced upon contact with sphingosine and regulates several genes involved in degradation of sphingolipids. Sphingolipids are extensively involved in the regulation of the immune system of the host. The reduced pathogenicity of the sphA mutant might also be a result of the inability to degrade sphingosine which is also known to exhibit antimicrobial activity [82].

The two component regulatory system comprising the sensor kinase CrbA and the response regulator CrbB is involved in carbon and nitrogen utilization but also impacts motility and production of pyoverdine in *P. aeruginosa*. *In vitro* studies revealed an enhanced phagocytic uptake of the *crbAB* system mutants by macrophages and neutrophils. Interestingly, when the single mutants were tested in an acute lung infection model or in a leukopenic peritonitis model, the *crbA*mutant showed a severe reduction in survival, while the *crbB* mutant behaved like the wild-type [83]. Thus, the *in vivo* experiments in the murine models revealed that CrbA most likely participates in cross talk with alternative response regulators besides CrbB.

An alternative regulatory system was studied by Li *et al.* [84]. They found one gene - *suhB*– to be strongly induced when wild-type bacteria were analyzed in the lung infection model. Apparently, SuhB regulated the expression of the type three secretion system (T3SS) by regulating the master regulator for T3SS - ExoA. Consequently, deletion of *suhB*results in downregulation of T3SS but concomitantly T6SS was upregulated and the bacteria exhibited a hyperbiofilm phenotype. Thus, *suhB* is involved in reciprocally regulating genes associated with either acute or chronic infection *in vivo*.

Despite its important role in bacterial pathogenicity, T3SS negative *P. aeruginosa* isolates can be recovered from patient material. In order to analyze whether T3SS deficient and sufficient bacteria might coexist, the importance of T3SS on *in vivo* survival of a heterogeneous bacterial population was studied [85]. T3SS-competent Wt and T3SS-ON (T3SS constitutively expressing) and –OFF (T3SS deleted) mutants were co-applied in the acute murine infection model. Interestingly, T3SS-deficient mutants out-competed their T3SS-positive counter parts. This might reflect social cheating behavior, where T3SS-OFF bacteria in direct neighborhood profit from T3SS-products of their sufficient partners. Curiously, these findings were only valid at a balanced ratio of T3SS-expressing and non-

expressing strains. Increasing the inoculum ratio in favor of the deficient population led to a loss of the effect. Similarly, the cheating effect was only found early during infection, while at later time points the outcompeting deficient population collapsed. This effect was lost in mice whose innate immune system could not respond to the bacterial infection. Clearly, survival strategies of *P. aeruginosa* do not follow a simple black-and-white principle, but are multi-layered and sophisticatedly entwined.

A novel two component system BfmS/BfmR regulating quorum sensing has recently been discovered [86]. BfmS is a sensor kinase which negatively regulates the response regulator BfmR. Deletion of *bfmS* leads to upregulation of *bfmR* and in turn to the upregulation of *rhlR* and the maturation of biofilms. Interestingly, BfmS can be converted by a single amino acid exchange into a positive regulator. Such mutations are found amongst clinical isolates from CF patients. However, whereas the deletion mutant of *bfmS* formed more mature biofilm, it showed reduced fitness in the acute murine lung infection model. Furthermore, despite the up-regulation of the *rhl* quorum sensing systems, which governs important virulence factors, the *bfmS*mutant bacteria showed reduced cytotoxicity against a murine lung epithelial cell line. In addition, phagocytosis of the mutants was enhanced. BfmS deletion had a large impact on the transcriptional profile and overall 3.6 % of the genome was affected. These included genes of the quorum sensing regulon, but also others which might explain the phenotype. In contrast, a quadruple mutant that affects quorum sensing by deletion of genes responsible for synthesizing the signaling molecules or their receptors (*lasIRrhlIR*) did not show a phenotype in the acute lung infection model. This is consistent with the idea that quorum sensing is only required in the chronic phase of the infection [87] and confirms the idea that the phenotype of the *bfmS* deletion is not due to the impact on quorum sensing.

Recently, mutants with a non-functional alp system, a prokaryotic cell lysis pathway [88], have been demonstrated to be unable to colonize murine lung in acute infection system. The alp system consists of the repressor AlpR that directly binds to the promoter of *alpA*. Upon DNA damage, AlpR is cleaved resulting in expression of the positive regulator *alpA* which triggers a cell lysis program. Apparently, the altruistic self-lysis of a fraction of the bacterial population improves the survival of the whole population possible by releasing nutrient or toxins. In congruence with this hypothesis, an equal mixture of wild-type and *alpA* mutants behaved like the wild-type in the infection system.

***In vivo* bacterial fitness.** Development of bacterial diversity and antibiotic resistance was tested in a combined nasal and pulmonary infection system [89]. *P. aeruginosa* was demonstrated to colonize the nasal cavities for more than 28 days, while bacteria were cleared from the lungs after 7days. However, the lungs were re-colonized by bacteria from the nasal cavity after 28 days. Although those isolates lost fitness they were more efficient in the colonization of the lung. Interestingly, some of those bacteria became resistant to antibiotic like tobramycin and ciprofloxacin. This suggests that the nasal tissues are an environment that favors the development of resistance and at the same time serve as a reservoir for well adapted bacterial populations that efficiently colonize the lung.

Murine lung infection models obviously serve well for the assessment of fitness costs of mutants. Accordingly, Roux *et al.*[90]tested mutants from a transposon library with insertions within genes known to be involved in antibiotics resistance. Surprisingly, such mutants displayed increased fitness *in vivo*. On the other hand, transposon integration into genes encoding intrinsic antibiotic resistance and thus their inactivation resulted in lower fitness. Similarly, gain of fitness was obtained when testing acquired or intrinsic antibiotic resistance in other pathogenic bacteria species. Thus, in

contrast to common belief, resistance against common antibiotics does not result in fitness costs for the bacteria.

Host factors. Mouse models can also be used to study the impact of host factors on the establishment and progression of infectious diseases. For instance, a severe infection and an increased inflammatory response were observed in mice in which the vascular endothelial growth factor (VEGF) was lung-specifically deleted [91]. VEGF is involved in maintaining the homeostasis of type II alveolar epithelial cells that are responsible for surfactant production. Similarly, the role of flagellin as immune stimulator was assessed in the acute murine infection model. Flagellin is recognized by epithelial and immune cells of the host via the cell surface toll like receptor 5 (TLR5) and the intracellular nod like receptor 4 (Nlrc4). Lung infection in mice in which both receptor genes had been deleted led to dramatically increased bacterial numbers and many of the mice succumbed to the infection. IL-1β and IL-18 levels were dramatically diminished in mice when Nlrc4 was deleted. This indicates that caspase 1 which is required for the secretion of these cytokines was activated. In addition, iNOS that is responsible for the production of NO was not upregulated in the double mutant. Thus, the two host receptors recognizing the bacterial molecular pattern flagellin trigger unique as well as redundant defense reactions against *P. aeruginosa*.

Development and evaluation of antimicrobialtherapies. The discovery of new bacterial pathogenicity pathways and metabolic adaptations to the infected site can pave the way for the development of new antimicrobial strategies that can be tested in the *in vivo* model systems. A study by Migiyama *et al.* [92] examined the *in vivo* efficacy of a quorum sensing inhibitor. They convincingly demonstrated in a mouse acute pneumonia model, that the AHL-lactonase AiiM attenuated *P. aeruginosa* virulence when expressed by the bacterial strain. Birkun *et al.* [93]tested the efficiency of a combined treatment therapy in an *in vivo* setting of acute bacterial lung infection. Using exogenous surfactant together with the aminoglycoside antibiotic amikacin they could observe antibacterial and anti-inflammatory effects in a rat pneumonia model and these beneficial effects were even more pronounced *in vivo* as compared to *in vitro*.

Mice, as experimental system for acute pneumonia may offer many advantages like financial aspects of purchasing, housing and care-taking or genetic diversity and modifications. On the other hand, the small size may complicate certain experimental procedures. For instance, pulmonary function testing (Spirometry, lung volume, diffusing capacity and oxygen desaturation) might be more convenient using rats rather than mice due to a larger body size. Furthermore, when assessing histopathologic scores the bigger organ size of rats may allow additional analyses. Such aspects led to the use of rats mainly for toxicological and pharmacological studies. In accordance, one well recognized and promising therapeutic strategy is the inhalation of nitric oxide gas. Various groups could show in a rat lung infection model that the inhalation of nitric oxide has beneficial effects on acute pneumonia outcome [94–98].

Chronic infections

The capability of *P. aeruginosa* to rapidly adapt to the environment of the host is central for the establishment of chronic infections. Whereas in acute infections a single cell status with full motility and the expression of a plethora of virulence genes is beneficial, during chronic infections the bacteria undergo a switch to a sessile population, which is characterized by the community-based production of a protective biofilm structure. In such structures, *P. aeruginosa* persists and becomes

insensitive against external assaults, including the activity of antibiotics or the immune effectors of the host. In consequence, studying *in vivo* models of biofilm physiology is a key aspect in understanding the armor of chronic *P. aeruginosa* infections. Only this will allow the development of desperately needed novel treatment strategies.

The infection of the lung of CF patients is one of the clinically most relevant chronic *P. aeruginosa* infections. To date a whole range of CF-associated mutations has been successfully introduced into the genome of mammals like pigs and mice [99,100].Initially, the recombinant mouse as a small animal model was hoped to mirror the human cystic fibrosis phenotype. However, the mutations lead only to intestinal disease manifestations. Only some recombinant mouse strains exhibited pulmonary abnormalities. Unfortunately, no phenotype was found to be equivalent to the CF lung disease in humans [100][101].Actually, a recent study by Shah *et al.* 2016 compares different clinical manifestations of CFTR mutations between mouse, pig and human and despite the problems highlights the great potential of the available animal model systems [102]. In addition, there are additional comprehensive reviews on CF animal models [25,103–106].However, an ideal animal model that fully mimics the different facets of CF as well as chronic biofilm-associated lung infections is currently not available.

Bead models. Most bacteria are quickly cleared from the lungs of healthy animals. Thus, for the establishment of chronic *P. aeruginosa* lung infections one of the biggest challenges is to retain a sufficient number of bacteria in the lungs over an extended time. A delayed clearance of bacteria from the respiratory tract of immune competent animals can be achieved by embedding them into an immobilizing extracellular matrix formed by agar, agarose or alginate from sea weed. This embedment efficiently protects the bacteria from the harsh host immune environment in the lung after their instillation via the nose or the trachea. This approach was published already by Cash *et al.* in 1979, who established the agar bead model in the ra t[107]. Since then a whole variety of "bead models" has been developed. Importantly, depending on the infecting strain, *P. aeruginosa* enmeshed in beads was observed to form macrocolonies inside and outside the beads within the bronchial lumen [108]. Macrocolonies are viewed as biofilm-related structures and therefore their presence supports one main requirement of a chronic infection model. Lemiuex *et al.* [109] used a sequence tagged transposon mutagenesis library to search for genes that are essential for *in vivo* survival of *P. aeruginosa* during agar bead lung infections in the rat. Interestingly, by a negative selection procedure, they found mutants with transposon insertions in prophages and genomic islands. Although the understanding of how the accessory genome impacts on fitness of *P. aeruginosa* during chronic infections may be still in its infancy, it may possibly impact on future therapeutic interventions.

For reasons of practicability and versatility, many researchers switched from the rat to the mouse as experimental animal to study the influence of single virulence and physiological factors on chronic lung infection of bead coated *P. aeruginosa*. Amongst the many siderophores that *P. aeruginosa* expresses, pyoverdin appears to be most important in the establishment of chronic lung infections. This is most likely not only due to the siderophore activity of pyoverdin but also due its virulence inducing capabilities [110]. In a more global approach, a library of sequence tagged mutants was screened for genes that impact chronic infections [111]. Obviously, to become chronic it is of advantage for *P. aeruginosa* to lose swimming and twitching motility, cut down on virulence genes like pyocyanin production and induce biofilm formation. Furthermore, clinical *P. aeruginosa* isolates recovered from early, intermediate and late stages of CF lung infection were tested in the murine

bead infection model [108]. Although all isolates were capable to establish chronic infections and to cause strong inflammation in the respiratory tract of the mice, early and intermediate isolates were highly lethal, whereas late isolates were attenuated. In a similar approach variants of a clinical isolate were compared [112]. Here, CF-adapted clones were able to shape the innate immune response in favor of their persistence. Interestingly, with such clones it was possible to maintain the chronic bead infection for three months. The mice showed hallmarks of CF-like airway remodeling and the establishment of fibrosis amongst many other CF symptoms. This could be considered as one step towards a more realistic CF/ chronic *P. aeruginosa* infection model.

The rodent models were also extensively used for the assessment of the therapeutic potential of novel compounds to fight chronic persistent infections. This included testing of the efficacy of a combination of anti-microbial bismuth-ethane-dithiol and tobramycin [113] in the rat model. Bacterial numbers in the lung were drastically reduced. Furthermore incorporation of the anti-microbial into liposomes effectively diminished the cytotoxic side effects as compared to the administration of free antibiotics. Similarly, the culture extract of a *Paenibacillus* isolate was shown to inhibit the quorum sensing regulated expression of the proteases LasA and LasB as well as production of pyoverdine. When the extract was orally administrated to rats which had received *P. aeruginosa* intratracheally in alginate beads, the number of bacteria was significantly reduced. The lung pathology was also milder [114]. Furthermore, L-Methionine could be shown to inhibit biofilm formation and to disrupt existing biofilms in the mouse model. Thereby, the upregulation of four DNases could be involved in biofilm disruption. Treatment of chronic infections with L-Methionine also rendered the bacteria susceptible to the activity of ciprofloxacin. Reassessment of the compound flucytosine, licensed for its use as an antifungal agent in the clinic, was found to exhibit an anti-pyoverdine activity by inhibiting the sigma factor PvdS, involved in pyoverdine expression. This resulted in an efficient protection of the mice after lethal challenge by *P. aeruginosa* in the chronic bead infection model [115].

The importance of host factors can be evaluated by the use of chronic mouse infection models. IL-17A is a known inflammatory cytokine. It could be shown that IL-17A is strongly and sustainably induced during chronic infections in mice [116]. This is in agreement with the presence of IL-17A in the CF lung [117]. In mice in which IL-17A or its receptor IL-17ra had been inactivated, the bacterial burden was found to be increased especially in the IL-17ra deficient mouse while in the IL-17A deficient mouse this was apparently compensated by up-regulation of IL-17F. Interestingly, depletion of IL17A during the chronic phase of the infection did not result in an increased bacterial burden but resulted in tolerance for the infection *i.e.* less infiltration of neutrophils and lower expression of the metalloprotease MMP9 involved in tissue reconstruction. Similarly, IL-22 deficient mice have been employed [118]. IL-22 is a cytokine that was shown to be involved in the mucosal host defense. No difference in the bacterial burden between the deficient and the wild-type mice could be observed. However, weight loss as a general indicator for animal health was only observed in the wild-type. Thus, IL-22 although not directly responsible for the defense reaction, is involved in the general inflammatory phenotype elicited in chronic *P. aeruginosa* infections.

A recent study by Li *et al.* [119] led to more comprehensive insights on the patho-adaptive phenotype displayed by *P. aeruginosa* during clinical phases of acute exacerbations. During such phases, bacteria likely disperse from the biofilm and undergo transition from a biofilm-associated to a motile, highly virulent mode of growth. It was shown that the virulence phenotype of disperser cells clearly differs from that of free-living planktonic cells. A BdlA mutant deficient for *in vitro*

dispersion was not capable of colonizing murine lungs in an acute infection model, but was out-competing the parental wild-type strain in the chronic bead infection model. Taken together those findings suggest a distinct virulence phenotype of disperser cells and convincingly show new aspects of fine tuning of bacterial pathogenicity during the infection process.

Tumor model. The establishment of chronic infections in the respiratory tract of the mice requires skilled handling and a professional experimental setup. Recently, we described an alternative model where bacteria colonize the tissue of subcutaneous murine tumors following intravenous or intraperitoneal instillation of the bacteria. Remarkably, *Salmonella enterica* serovar Typhimurium is able to form biofilms in these tumors [120]. Similarly, *P. aeruginosa* forms bacterial clusters embedded in a biofilm matrix within the tumor tissue, which depends on a functional quorum sensing system [121]. Neutrophils are attracted to the site of bacterial colonization. However, bacteria are rarely observed within such phagocytes. At first sight, tumors and the lung are apparently highly diverse tissues. However, the environmental niche that the bacteria encounter within the tumor seem to be very similar to that of a chronically infected CF lung or that of burn wounds, as the bacterial transcriptome reviled [122,123]. The tumor model thus provides a simple model to investigate physiological factors involved in biofilm formation or the influence of a co-infection (see below). Furthermore, the tumor model seems to be a valid and simplified system for research on the efficacy of antimicrobial compounds and novel treatment regimens [124].

Polymicrobial studies

P. aeruginosa is the most dominant bacterial pathogen recovered from chronically infected CF lungs. However, there are other pathogens such as *Haemophilus influenza*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cenocepacia*. The relationship of these colonizers to each other may be of cooperative or competitive nature. Although intensive *in vitro* studies have generated knowledge on the polymicrobial interrelationships, many of the specific bacterial interactions only become apparent under *in vivo* infection conditions.

Over 50 % of under two year old CF patients carry *S. aureus* in their lungs (CF foundation annual report), whereas colonization with *P. aeruginosa* usually occurs later. Nevertheless, both pathogens can co-colonize the respiratory tract over extensive time periods. Interestingly, *P. aeruginosa* isolates from acute CF infections strongly outcompeted *S. aureus in vitro* as well as in the acute mouse model [125]. In contrast, *P. aeruginosa* isolates from the late disease stages had lost this property, in agreement with the finding that *P. aeruginosa* downregulates virulence during chronic infections.

The ability to outcompete *S. aureus* is obviously dependent on *P. aeruginosa´s* capability to sense the presence of *S. aureus*. *In vitro*, in a murine chronic wound model as well as in the drosophila crop model the *P. aeruginosa*PA0601gene was found to be crucial for the expression of this phenotype. Using a transposon library PA0601 was identified as a sensor for N-acetylglucosamin, a component of the peptidoglycan of gram-positive bacteria [126]. PA0601 appears to be the regulator of a two component system. Its stimulation resulted in the secretion of pyocyanin and elastase as well as the expression of genes involved in the biosynthesis of a quorum sensing signaling molecule [Pseudomonas quinolone signal (PQS)], which resulted in enhanced host tissue damage.

Burkholderia cenocepacia, an opportunistic Gram-negative bacterium is often isolated from the sputum CF patients being in their early 20s and older. Most of those patients already have a long history of chronic *P. aeruginosa* infections, often for more than a decade or two. Little is known about the inter-species interaction of *P. aeruginosa* and *B. cenocepacia in vivo*. However, by the use of the mouse chronic infection model, it was demonstrated that a mixed infection leads to an increased inflammatory response in the host [127] and *B. cenocepacia*was outcompeted by *P. aeruginosa*. Furthermore, the presence of *B. cenocepacia* in the mixture triggered a higher biofilm production within the *P. aeruginosa* population.

The oxygen-limited or even –free microenvironment of the thick airway mucus is also home to certain anaerobic species of the genera Prevotella, Propionibacterium, Actinomyces and Veillonella. To date, the role of strict anaerobes in the CF lung disease is not clear. With the aim to explore whether *P. aeruginosa* may be influencedby *V.parvula* during chronic infections, a bacterial mixed infection was established in chronic infection tumor model [128].*V. parvula* enhanced the overall bacterial burden of *P. aeruginosa*as compared to single *P. aeruginosa* infections and increased the inflammatory reaction. The presence of *V parvula* might stimulate growth of *P. aeruginosa* or simple enlarge the niche for colonization. *V. parvula* appears not to benefit from the *P. aeruginosa* co-infection. In general, it seems that *P. aeruginosa* profits the most from the altered host niche during co-infections. This may explain why *P. aeruginosa* at the end dominates the CF lung.

Combining different animal models

Combining different animal models may prove beneficial. Screenings of putative new antimicrobial compounds in a non-vertebrate animal model may give first information on efficacy and toxicity. Promising candidates may then be carried further to the more complex mammalian models. For example, Palliyil *et al.* [129]evaluated the in vivo efficacy of an antibody targeting a quorum sensing signaling molecule in *C. elegans* prior to testing it in a mouse infection model. In both models a protective effect was observed as well as prolonged survival after infection and treatment.

Dubern *et al.* [130]performed a comparative study and tested the pathogenicity of various *P. aeruginosa* transposon mutants in very diverse animal models. Their multi-host approach included *in vitro* cell culture, *C. elegans, D. melanogaster* and a mouse model. For some mutants they found agreement in the virulence profile between the models, whereas for other mutants they observed differences. Similarly, Hilker *et al.* [131] compared various clonal *P. aeruginosa* isolates in various infection models including Galleria and the mouse. They also observed extreme differences in infection outcome. It thus seems that some virulence determinants are host specific. Interestingly, late CF isolates, in which the homogenisate-1-2-dioxginase was downregulated, presented an increased virulence in *C. elegans* while in the mouse the virulence apparently was reduced [132]. This demonstrates the power of combining different *in vivo* infection models towards a more integrated view of the pathogenic potential of single *P. aeruginosa* clones or mutants, clinical isolates or whole populations.

Conclusion

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* presents itself as an extremely complex microorganism. It is able to infect most divergent hosts ranging from plants to immune

competent humans. To be able to do so, it is equipped with a vast number of sensory systems that allow the pathogen to recognize precisely its surroundings and react appropriately. In addition to infecting a very broad host range, *P. aeruginosa* is capable of changing from a highly virulent planktonic mode of growth as presented during acute infections to a sessile biofilm-associated growth stage where the microorganisms become almost invulnerable. This might explain why despite aggressive antibiotics treatment, patients with cystic fibrosis will eventually succumb to the chronic persistent infections.

Finding a simple solution that provides efficient treatment strategies against such bacterial infections is seriously hampered. Nevertheless, as described in the present review attempts are made that may eventually solve these problems in invertebrate (Fig. 1) and vertebrate (Fig. 2) animal models in accordance with their respective strengths. Transposon libraries were employed to understand bacterial behavior during an infection process. Similarly, clinical isolates have been sequenced and their particular properties characterized. Advanced molecular microbiology allows efficient generation of mutants that lack a particular gene or function and the characterization of such mutants *in vitro* will reveal regulatory circuits and the molecular and physiological context of bacterial pathogenicity. However, ultimately such data need to be confirmed *in vivo*. Several state of the art experimental animal systems are available as described here. Especially, the mouse chronic system was shown to more and more mimic the human conditions in the infected CF lung. Thus despite of the obstacles described above, advanced experimentation combined with systems infection biology will eventually yield a complete picture of chronic *P. aeruginosa* infections. The divergent genes of clinical strains isolated from a given patient may then function as biomarkers for the prediction of disease outcome and also individualized treatment.

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