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INVESTIGATING THE ROLE OF *PSEUDOMONAS AERUGINOSA* METABOLISM IN VIRULENCE

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STAVRIA PANAYIDOU

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STAVRIA PANAYIDOU

A Dissertation Submitted to the University of Cyprus in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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STAVRIA PANAYIDOU

STAVRIA PANAYIDOU

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Doctoral Candidate: Stavria Panayidou

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Examination Committee:

Research Supervisor:

Yiorgos Apidianakis, Assistant Professor ___________________________________

Committee Member:

Chrysoula Pitsouli, Assistant Professor ___________________________________

Committee Member:

Katerina Strati, Assistant Professor ___________________________________

Committee Member:

Dimitris Tsaltas, Associate Professor ___________________________________

Committee Member:

Shimshon Belkin, Professor ___________________________________

STAVRIA PANAYIDOU

DECLARATION OF DOCTORAL CANDIDATE

The present doctoral dissertation was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy of the University of Cyprus. It is a product of original work of my own, unless otherwise mentioned through references, notes, or any other statements.

Stavria Panayidou ……………....[Full Name of Doctoral Candidate]

…………………………………..[Signature]

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ΠΕΡΙΛΗΨΗ

Η τρέχουσα παγκόσμια τάση αντοχής στα αντιβιοτικά απαιτεί νέες προσεγγίσεις για αντιμολυσματική θεραπεία. Η Ψευδομονάδα (*Pseudomonas aeruginosa*), είναι ένα Gram-αρνητικό βακτήριο που ταξινομείται μεταξύ των παθογόνων πρώτης προτεραιότητας για την ανάγκη εξεύρεσης νέων και αποτελεσματικών θεραπειών. Σε αυτή την εργασία, επιδιώκοντας να εντοπίσουμε νέους θεραπευτικούς στόχους έναντι της *P. aeruginosa*, επικεντρωθήκαμε σε γονίδια του μεταβολισμού του βακτηρίου που σχετίζονται με τη λοιμογόνο δράση του, τα οποία δεν είναι απαραίτητα για τη φυσιολογική ανάπτυξή του. Για να εντοπίσουμε γονίδια που σχετίζονται με τη λοιμογόνο δράση του στελέχους PA14 της *P. aeruginosa*, αξιολογήσαμε 553 μεταβολικές και 95 μη μεταβολικές γονιδιακές μεταλλάξεις αυτού, στη Δροσόφιλα (*Drosophila melanogaster*) και βρήκαμε ότι το 16.5% των μεταβολικών και το 8.5% των μη μεταβολικών γονιδίων, απαιτούνται για τη πλήρη λοιμογόνο δράση του εν λόγω βακτηρίου. Ακολούθως παρατηρήσαμε ότι το 11.8% από τα επιλεγμένα στελέχη που φέρουν μεταλλάξεις σε μεταβολικά γονίδια, καθώς και όλα τα στελέχη που φέρουν μεταλλάξεις σε μη μεταβολικά γονίδια, αναπτύσσονται αποτελεσματικά στη βακτηριακή καλλιέργεια ή αποικίζουν τον ξενιστή κατά παρόμοιο τρόπο με το στέλεχος αγρίου τύπου. Επομένως, ένα σημαντικό ποσοστό των στελεχών που φέρουν μεταλλάξεις σε γονίδια του μεταβολισμού εμφανίζουν εξασθενημένη λοιμογόνο δράση, η οποία δε μπορεί να αποδοθεί σε αυξοτροφία. Τα εν λόγω γονίδια, ανήκουν σε 7 κύρια μεταβολικά μονοπάτια και τα στελέχη που φέρουν τις αντίστοιχες μεταλλάξεις εμφανίζουν μειωμένες ιδιότητες λοιμογόνου δράσης, καθώς και εξασθένηση σε μοντέλο οξείας επιμόλυνσης ποντικών.

Επιπλέον, αξιολογήσαμε ποσοτικά την παθογονικότητα 18 πλήρως αλληλουχημένων στελεχών της *P. aeruginosa* και 12 πλήρως αλληλουχημένων στελεχών μη-*P. aeruginosa* σε δύο δοκιμασίες επιμόλυνσης στη Δροσόφιλα, και 6 από αυτά επικυρώθηκαν σε μοντέλο οξείας επιμόλυνσης ποντικών. Συγκριτική γονιδιωματική ανάλυση όλων των στελεχών δεν καταδεικνύει κάποια συσχέτιση μεταξύ της παθογονικότητας και του γονιδιακού περιεχομένου διαφορετικών στελεχών *Pseudomonas*. Επομένως, χρησιμοποιήσαμε μεταγραφωμική προσέγγιση με την οποία συγκρίναμε το μεταγράφημα $3^{ων}$ υψηλών και $3^{ων}$ χαμηλών σε παθογονικότητα στελεχών. Ανακαλύψαμε ότι η λοιμογόνος δράση της *P. aeruginosa*, η οποία μέχρι στιγμής παραμένει απρόβλεπτα συνδυαστική σε επίπεδο γονιδιώματος, μπορεί να περιγραφεί σε μεταγραφικό και λειτουργικό επίπεδο από συντηρημένα στοιχεία του μεταβολισμού, τα οποία ελέγχουν και υποδεικνύουν τη λοιμογόνο δράση σε διαφορετικά παθογόνα στελέχη *P. aeruginosa*.

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ABSTRACT

The current worldwide spread of antibiotic resistance demands novel approaches for anti-infective therapy. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative bacterium classified among the few priority pathogens urgently requiring new and effective treatments. In this study to identify novel therapeutic targets against *P. aeruginosa*, we focused on virulence-related metabolic genes, which are not essential for physiological bacterial growth. By assessing 553 metabolic and 95 non-metabolic gene mutants of the *P. aeruginosa* strain PA14 for virulence in *Drosophila melanogaster*, we found 16.5% of the metabolic and 8.5% of the non-metabolic genes to be important for full virulence. Strikingly, 11.8% of the selected metabolic and all the non-metabolic mutants grow efficiently in culture or colonize the host like the wild-type strain. Thus, a significant portion of the metabolic mutants, exhibit defects in virulence that cannot be attributed to auxotrophy. The identified metabolic genes belong to 7 central metabolic pathways and their mutants exhibit defects in various virulence properties, as well as in an acute murine lung infection assay.

Moreover, we quantitively assessed the pathogenicity of 18 *P. aeruginosa* and 12 non-*P. aeruginosa* fully sequenced strains in two *Drosophila* infection assays, and six strains were validated in a mouse infection assay. Comparative genomic analysis of all strains shows no correlation between pathogenicity and gene content of different *Pseudomonas* strains. For this reason, we used a transcriptomic approach by which we made a comparison between the transcriptome and the virulence potential of 3 high and 3 low in virulence *P. aeruginosa* strains. We found that *P. aeruginosa* virulence, which to this point remains unpredictably combinatorial at the genome level, may be described at the transcriptome and functional level by conserved core-metabolism modules that control and indicate the virulence of disparate *P. aeruginosa* strains.

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DEDICATION

Ἡ Ἰθάκη σ' ἔδωσε τ' ὡραῖο ταξίδι.
Χωρὶς αὐτὴν δὲν θά 'βγαινες στὸν δρόμο.
Ἄλλα δὲν ἔχει νὰ σὲ δώσει πιά.

Κι ἂν πτωχικὴ τὴν βρεῖς, ἡ Ἰθάκη δὲν σὲ γέλασε.
Ἔτσι σοφὸς ποὺ ἔγινες, μὲ τόση πεῖρα,
ἤδη θὰ τὸ κατάλαβες οἱ Ἰθάκες τὶ σημαίνουν.

Κ. Π. Καβάφης

Στα χρόνια

που αφιέρωσα σ' αυτό το ταξίδι !

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ABBREVIATIONS

ADPRT	ADP-ribosyltransferase
AI1	Autoinducer 1
AI2	Autoinducer 2
AHLs	Acyl Homoserine Lactones
AMPs	Antimicrobial Peptides
CF	Cystic Fibrosis
CFUs	Colony-Forming Units
CCR	Carbon Catabolite Repression
cDNA	complementary DNA
CHD1	Chromo Helicase Domain Protein 1
Crc	Catabolite repression control
DGCs	Diguanylate Cyclases
eDNA	extracellular DNA
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
EPS	Extracellular Polymeric Substances
FGAR	5-phospho-ribosyl-N-formylglycinamide
GAP	GTPase Activating Protein
GAR	5-phospho-ribosyl-glycinamide
IL-6	Interleukin-6
IL-17	Interleukin-17
IL-27	Interleukin-27
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny Broth

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LPS	Lipopolysaccharide
LT50%	Lethal Time 50%
MvfR	Multiple virulence factor Regulator
M9	Minimal media M9
NF-κB	Nuclear Factor kappa B
OD_{600nm}	Optical Density at 600 nm
PDEs	Phosphodiesterases
PQS	Pseudomonas Quinolone Signal
PON1	Paraoxonase-1
PutA	Proline utilization A
QS	Quorum-Sensing
RBS	Ribosomal Binding Site
RLs	Rhamnolipids
TCS	Two-Component System
THF	Tetrahydrofolate
Tn-mutant	Transposon insertion mutant
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
WT	Wild-Type
VFs	Virulence Factors

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CHAPTER 1

INTRODUCTION

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1.1 *P. aeruginosa*: Classification and Morphology

Classified in the phylum of Proteobacteria, class of Gammaproteobacteria, order Pseudomonadales and family Pseudomonadaceae, the genus *Pseudomonas* includes some of the most ubiquitous and diverse bacterial species in nature able to utilize a wide range of organic compounds and colonize a wide range of niches. Among the members of this genus, the heterotrophic Gram-negative aerobic bacterium *P. aeruginosa* is remarkable for its capacity to inhabit diverse environments, including soil and water, and infect multiple organisms, such as insects, plants and animals [1-6]. Its morphological characteristics facilitate the movement across several surfaces since is monoflagellated and rod-shaped, measuring about 1.5-5.0 μm in length and 0.5-1.0 μm in diameter [7]. *P. aeruginosa* is an important opportunistic human pathogen inflicting predominantly burned, cystic fibrosis (CF) and otherwise immunocompromised patients. It is a frequent cause of nosocomial infections being the most common pathogen isolated from patients who have been hospitalized longer than 1 week. The first reason for its high prevalence is its high virulence repertoire, which includes biofilm formation and quorum-sensing signaling networks [8, 9]. Additionally, it is a foodborne pathogen – found, for example, in hospital water, food, and feeding tubes – and an efficient intestinal colonizer, especially upon antibiotic treatment and surgical stress [10].

1.2 Signaling systems that regulate virulence in *P. aeruginosa*

The identification of new targets for drug development to fight microbial infections is a challenge for anti-infective drug discovery. A staggering amount of money is spent every year in this field of research. It is now well-known that the virulence, the antibiotic resistance as well the biofilm formation of many bacterial pathogens is mainly controlled by quorum-sensing (QS), a cell to cell communication mechanism that coordinates gene expression [11, 12]. Since many pathogens are resistant to antibiotics, the quorum-sensing has emerged as a promising target for disrupting pathogenesis in many organisms including humans [2]. For the activation of the QS systems, a certain bacterial cell density is required and a threshold concentration of signaling molecules must be achieved [13]. *P. aeruginosa* is considered an infamous human opportunistic pathogen not only because it is highly virulent, but also due to its low susceptibility to antibiotics [14].

Gram-negative bacteria including *P. aeruginosa*, mostly use N-Acyl homoserine lactones (AHLs) as communication molecules [13]. There are two well-defined AHL quorum-sensing systems in *P. aeruginosa*: the *las* and *rhl* systems. The transcriptional activators of these systems are the LasI/LasR and RhlI/RhlR respectively [13]. LasI synthase produces the N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL) also known as autoinducer 1 (AI1), a

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signaling molecule (ligand) that makes a complex with the transcriptional regulator LasR (receptor). This leads to the upregulation of the transcription of many genes, in a cell density-dependent manner [13]. The LasR/3-oxo-C_{12}-HSL complex regulates itself since it upregulates the expression of *lasI* and enhances its activity. It also induces the transcriptional regulator RsaL, which is a negative regulator of *lasI* expression (**Figure 1**) [15, 16]. Moreover, the LasR/3-oxo-C_{12}-HSL complex triggers the transcription of *rhlR* and *rhlI* and consequently activates the *rhl* system (**Figure 1**) [13,17]. Similarly, to the *las* system, the activation of *rhl* system requires the binding of the autoinducer 2 (AI2) called N-butanoyl-L-homoserine lactone (C_4-HSL), to the receptor RhlR. AI2 is produced by the RhlI synthase [13]. Since AI1 and AI2 are required for QS activation, many studies are focused on the discovery of AI1 and AI2 antagonists capable to inhibit LasR and RhlR. The compound 3-oxo-C_{12}-(2-aminocyclohexanone), also known as N-(2-oxocyclohexyl)-3-oxododecanamide, is an analog of the AI1 (3-oxo-C_{12}-HSL), and it inhibits LasR/AI1-dependent activation of transcription by binding LasR, with the same affinity as AI1, but without activating it [18]. This action results in the inhibition of LasI and RhlI, as well as in the reduced production of virulence factors (pyocyanin and elastase B) and biofilm [18]. There is a possibility for AI1 to also antagonizes RhlR/AI2 interaction by binding to RhlR [18].
Apart from the classical AHL Gram-negative signals, *P. aeruginosa* has another well-defined signaling molecule, termed the *Pseudomonas* quinolone signal (PQS) [13,19]. Similarly, to the previous QS systems, the PQS-regulated production of virulence factors requires a certain threshold concentration of this molecule [13,19]. When this happens, PQS binds to its receptor PqsR, commonly known as 'multiple virulence factor regulator' MvfR and together bind to the promoter region for activating the transcription of the target genes [13,19]. The levels of PQS are positively controlled by the LasR/3-oxo-C_{12}-HSL complex, by binding to the promoter region of the MvfR (**Figure 1**) [19]. On the contrary, the RhlR/N-butanoyl-L-homoserine lactone (C_4-HSL) complex represses the production of the PQS signal by interfering with *pqsR* and *pqsABCDE* promoters of the *pqs* system (**Figure 1**) [17, 20]. Interestingly, PQS, apart from being a signaling molecule and regulator of virulence genes, mediates the formation of the membrane vesicles of the bacterial cells, by stimulating the lipoposaccharide (LPS) to form liposome-like structures [21]. Lipopolysaccharide (LPS) is the major component of the outer membrane of the Gram-negative bacteria [22]. According to recent *in vitro* studies, LPS expression is also regulated by both *las* and *rhl* QS systems [23]. However, the effect in LPS production was more obvious in the *lasI/rhlI* and *lasR/rhlR* double mutant strains, than in *lasI* and *lasR* single mutant strains indicating that both QS systems are required for the efficient LPS production [23].

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Conclusively, the quorum-sensing systems are crucial for the bacterial viability, survival and persistence, thus they might be effective targets for drug development.

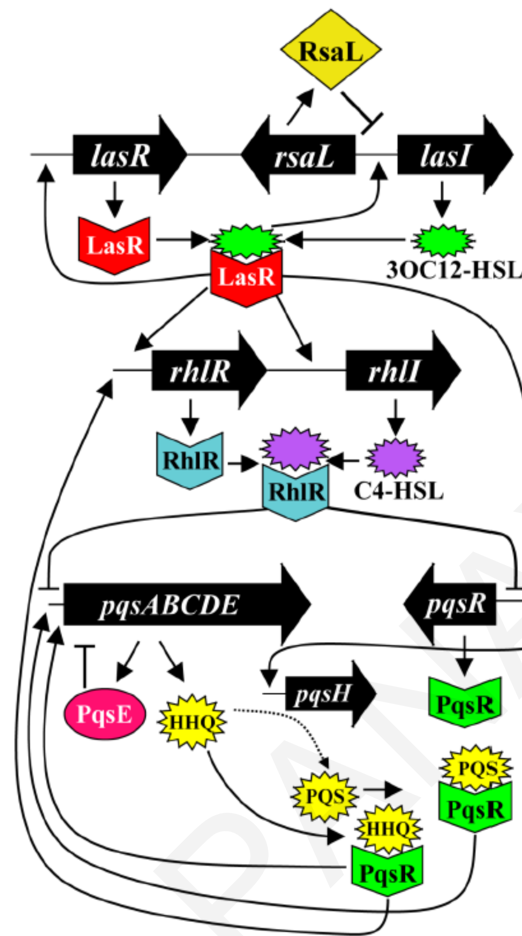

Figure 1. The QS network of *P. aeruginosa*. It consists of three well-studied systems, namely *las*, *rhl,* and *pqs*. The *las* system is the first QS system to be activated in a rich growth medium. Activated LasR can activate the transcription of *rhlR*, *rhlI*, *pqsR* and *pqsH*. The transcriptional regulator RsaL acts as a negative regulator of the *lasI* expression. The activated RhlR negatively regulates the *pqs* system by repressing the activity of *pqsR* and *pqsABCDE* promoters. Studies have also shown that the *pqs* system positively regulates the *rhl* system [17].

1.3 Acute and Chronic Infection of *P. aeruginosa*

The infections caused by the opportunistic human pathogen *P. aeruginosa* can be acute or chronic. The acute infections are characterized by rapid tissue damage and sepsis leading to high mortality rates in a short amount of time [24]. On the other hand, chronic infections usually persist for long periods and in some cases last for years [24]. Obviously, the bacterial mechanisms underlying each type of infection as well as the array of virulence factors that

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are implicated in each case are very different. In acute infections, bacteria adopt a planktonic lifestyle, while chronic infections are more related to a biofilm lifestyle. For example, acute infection initiates by binding of free-living bacteria to epithelial cells triggering a host inflammatory response [25, 26]. This occurs through two major adhesins of *P. aeruginosa*, type IV pili and flagella [25, 26]. Once in contact with the epithelial cells, *P. aeruginosa* utilizes its type III secretion system (T3SS) to enhance the pathogenic process by injecting effector proteins into the host cells [27, 28]. The T3SS is one of the protein secretion systems of *P. aeruginosa* that enables its survival in hostile environments and facilitates the colonization of eukaryotic and other hosts. The T3SS enhances the disease severity in many animal models, including acute pneumonia models, because it plays an important role in *P. aeruginosa* survival in the blood and in its rapid dissemination in the bloodstream, leading to the host death within hours or days [27, 28]. The T3SS effector proteins inhibit phagocytosis and act synergistically with LasB protease to disrupt the endothelial barrier integrity allowing the bacteria entrance to the bloodstream and dissemination throughout the host body (**Figure 2**) [26, 29]. On the other hand, in chronic infection, bacteria grow slowly and are less cytotoxic due to loss of the determinants of the free-living bacteria, including motility and functional T3SS [26, 29]. In chronic infections, there is a conversion from planktonic to a mucoid phenotype that facilitates the persistent of the bacteria in the host for long periods without reaching the bloodstream, a situation that is common in the lungs of patients with cystic fibrosis (CF) [26, 29]. The mucoid phenotype of *P. aeruginosa* strains, in chronic infections, is a result of overexpression of alginate usually due to mutations within the *mucA* gene [30]. Interestingly, mutations in the *mucA* gene were also connected with the suppression of T3SS genes [30]. However, *P. aeruginosa* has also a different type of secretion system, called as type VI secretion system (T6SS), which significantly contributes to the successful colonization and persistence of this bacterium in the lungs of cystic fibrosis patients [31]. Finally, the GacS/GacA two-component system is crucial for the switch from an acute to a chronic infection mode in *P. aeruginosa* (**Figure 3**) [26]. The GacS/GacA regulates this lifestyle transition by inducing the transcription of genes encoding the two regulatory small RNAs, RsmY and RsmZ [26].

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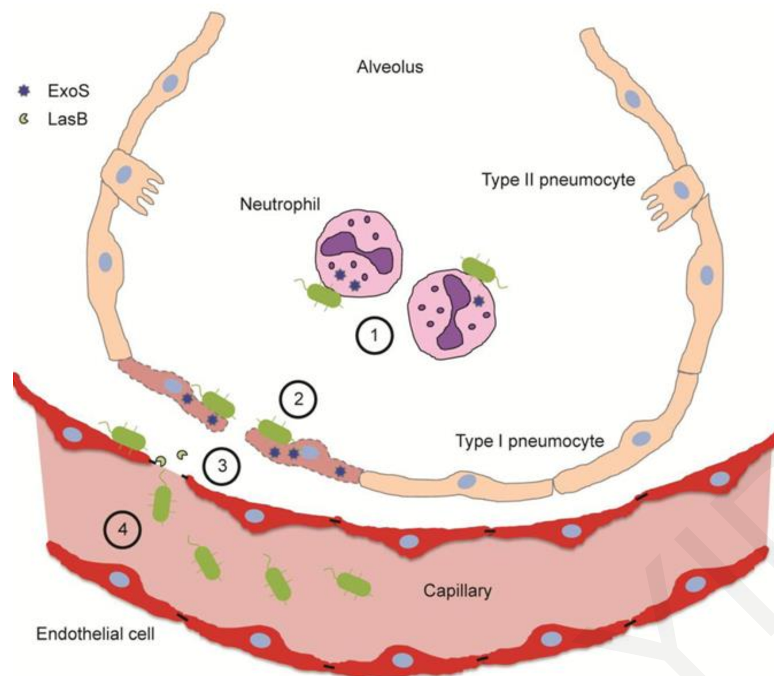

Figure 2. *P. aeruginosa* dissemination from the human lungs to the bloodstream. (1) Bacterial entry to the airways and recruitment of the neutrophils in the alveolus. *P. aeruginosa* blocks phagocytosis by injecting ExoS, a T3SS effector protein, into the neutrophils. (2) *P. aeruginosa* binds to the airway epithelial cells and injects *ExoS* to the Type I pneumocytes leading to the disruption of the epithelial barrier. (3) The protease LasB disrupts the endothelial barrier. (4) *P. aeruginosa* passes to the bloodstream and disseminates throughout the body [29].

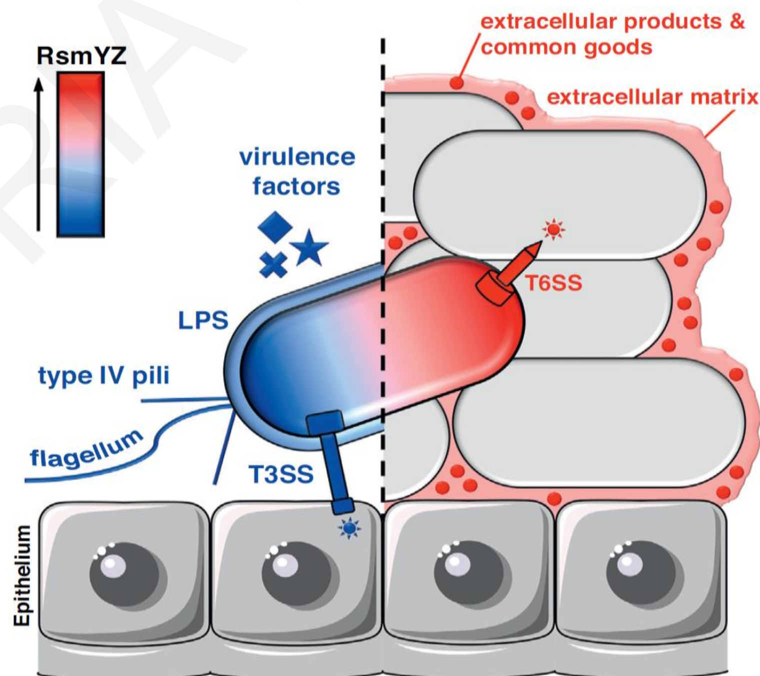

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Figure 3. Regulation of lifestyle switch in *P. aeruginosa* by the small RNAs RsmY/Z. Low levels of these small regulatory RNAs (blue, left) in *P. aeruginosa*, are associated with a planktonic lifestyle and acute infections. This type of bacterial lifestyle is characterized by increased motility mediated by rotating flagella and type IV pili, the release of virulence factors such as T3SS effector proteins and the presence of LPS. High levels of the RsmY/Z (red, right) are associated with a sessile lifestyle and chronic infections. This type of lifestyle is mainly characterized by the activity of the T6SS and the formation of biofilm [26].

1.3.1 Type III Secretion System (T3SS)

The T3SS is a complex protein and translocation machinery which, as described above, has a critical role in *P. aeruginosa* acute infections [28, 32]. It consists of five operons, that encode 5 types of proteins with different roles and functions including, proteins that comprise the injectisome responsible for the transport of T3SS specific proteins from the bacterial cytosol to the extracellular environment; the translocation apparatus which consists of proteins that regulate the translocation of specific T3SS proteins into the host cells; regulatory proteins responsible for the secretion process; effector proteins which are the proteins that are injected into the host cells during infection as well as chaperone proteins which facilitate the secretion of their cognate protein partners [28, 32]. In *P. aeruginosa* strains, only four effector proteins have been identified (ExoT, ExoS, ExoU, ExoY), while most of the *P. aeruginosa* strains do not have a complete set of effector-encoding genes [33]. For example, only the *exoT*, *exoU* and *exoY* are encoded in the genome of *P. aeruginosa* strain PA14 [34]. Importantly, *exoT* is present in most of the virulent *P. aeruginosa* clinical isolates while, the other three effector-encoding genes, are encoded only in some of the clinical strains, suggesting a critical role of the ExoT protein in *P. aeruginosa* pathogenesis [35]. ExoT and ExoS are homologous bifunctional proteins [28, 35]. Both have an N-terminal GTPase activating protein (GAP) domain and a C-terminal ADP-ribosyltransferase (ADPRT) domain [28, 35]. Studies have shown that, with the ADPRT and GAP domain, ExoT can induce apoptosis in a variety of target host cells while, ExoS inhibits phagocytosis and promotes apoptosis and bacterial dissemination [36, 37]. ExoU is a potent phospholipase that can rapidly cause necrosis of eukaryotic cells, thus consequently promotes *P. aeruginosa* persistence and dissemination [28, 35]. ExoY, is an adenylate cyclase that promotes the accumulation of cyclic nucleotides in the host cells [38]. It was recently found that ExoY promotes an increase in the cyclic UMP levels during acute mouse lung infection, however, how this relates to the pathophysiologic changes, remains unknown [38]. The expression of T3SS is regulated transcriptionally and post-transcriptionally in response to host cell contact and environmental Ca^{2+} levels.

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1.3.2 Type VI Secretion System (T6SS)

About 25% of Gram-negative bacteria species, including *P. aeruginosa*, have type VI secretion system (T6SS) [39, 40]. The structure of this system is similar to the puncturing device of bacteriophages promoting a contact-mediated-delivery activity [39, 40]. The T6SS gives a survival advantage to *P. aeruginosa* by secreting toxins into neighboring bacteria promoting cell lysis and inhibition of bacterial growth, but also by translocating protein effectors into the host cells [39, 40].

P. aeruginosa encodes three T6SSs, H1-, H2- and H3-T6SS, that most likely have been acquired by horizontal gene transfer, since they have separate evolutionary histories [39].

The H1-T6SS plays an important role in chronic infections; it was found active in chronically-infected cystic fibrosis patients while mutants in T6SS genes failed to replicate efficiently in rat models of chronic respiratory infections [41]. The H1-T6SS also participates in biofilm formation of *P. aeruginosa* because it is co-regulated with other virulence factors that are involved in chronic infection including biofilm-associated genes [41]. Although H2- and H3-T6SS interact with both prokaryotic and eukaryotic cells, the H1-T6SS seems to be important for bacterial competition in the same niche [41, 42].

There are three categories of T6SS genes: (a) genes encoding membrane-associated proteins, (b) genes related to tailed bacteriophage components including, its syringe components e.g. Hcp and VgrG and components that provide energy e.g. ClpV. In the third category (c) are genes with unknown function [39]. ClpV is an ATPase with important roles in T6SS-regulated virulence while is also important for the disassembly of the bacteriophage sheath and the secretion of Hcp [40]. VgrG and Hcp proteins are considered as effectors of the T6SS since they are secreted by this system [39]. However, Hcp is also a chaperone and receptor of the other T6SS effectors [39].

1.3.3 *P. aeruginosa* motility

P. aeruginosa uses three different types of motility: swarming, swimming and twitching that potentially facilitate the colonization in several environmental niches [43]. Swimming is individual movement in liquid and is mediated through rotating flagella, while twitching occurs on solid surfaces by using type IV pili [43]. It was recently demonstrated that *P. aeruginosa* controls twitching motility by sequential control of the type IV pili movements [44]. Swarming is a more complex type of motility required for the coordinated and rapid movement of a bacterial population on a semi-solid surface [43, 45]. Swarming requires both flagella and type IV pili [43]. Swarming *P. aeruginosa* cells secrete rhamnolipids (RLs), which reduce the surface tension and modulate the movement (**Figure 4**) [43, 46]. The RLs production

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is regulated through the quorum-sensing and specifically by the Rhl system in a cell density-dependent manner [43].

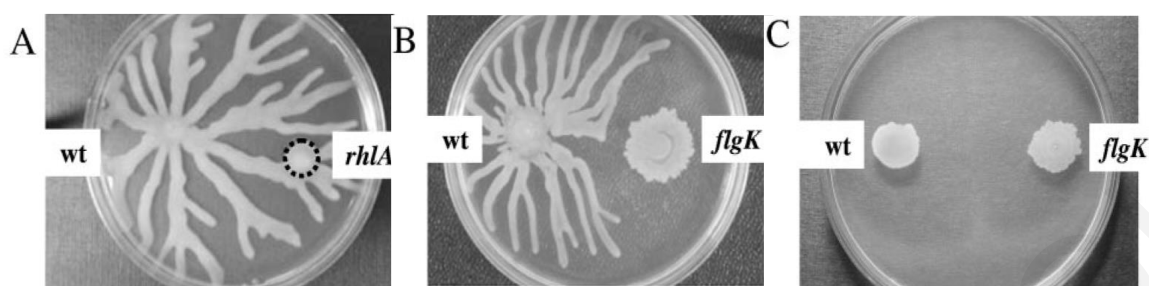

Figure 4. Rhamnolipids modulate the swarming motility. (**A**) Swarming motility of WT and *rhlA* mutant cells on 0.5% agar plates for 16 hours. The *rhlA* mutant cells have a non-swarming phenotype; the swarming motility of the WT is not affected by the presence of the *rhlA* mutant. (**B**) Swarming motility of WT and *flgK* mutant on 0.5% agar plates for 16 hours. The production of rhamnolipids from the non-swarming *flgK* mutant, affect the movement of the WT; the tendrils of the WT, change direction and does not contact the *flgK* mutant. (**C**) Swarming motility of WT and *flgK* mutant on 0.5% agar plates containing purified rhamnolipids. The swarming motility of the WT was repressed by the purified rhamnolipids and after 16 hours a non-swarming phenotype was observed [46].

1.3.4 Biofilm formation in *P. aeruginosa*

Biofilms are organized communities of bacteria embedded in extracellular polymeric substances (EPS) that provide protection from the outside environment, as well as antibiotic resistance [47, 48]. To form a biofilm, bacteria lose their motility, attach to surfaces and form aggregations [47]. Biofilm formation provides a survival advantage to *P. aeruginosa*, since a sessile lifestyle with low virulence prevents the detection by the host immune system and protects the bacterial population from environmental stresses [47]. There are at least three polysaccharides (alginate, Pel, Psl), which are important for the stability of *P. aeruginosa* biofilm while, the extracellular DNA (eDNA) is also determinant for the biofilm architecture [47, 48]. The overproduction of alginate leads to a mucoid phenotype, which is a hallmark of chronic infections [47]. It was recently demonstrated that the production of alginate by mucoid strains of *P. aeruginosa* facilitates the coinfection with *S. aureus* by inhibiting several antimicrobial agents produced by *P. aeruginosa* [49]. Consequently, the biofilm lifestyle enables the coexistence of different bacterial species with antibiotic resistance, promoting chronic infections.

The regulation of biofilm formation is post-transcriptionally regulated by a small molecule known as cyclic-3'5'-diguanylic acid (cyclic di-GMP) [13, 47]. Cyclic di-GMP mainly controls

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the switch between motility and sessile state in a concentration-dependent manner [13, 47]. For example, elevated levels of cyclic di-GMP are related to inhibition of motility and formation of biofilm (**Figure 5**) [13, 47]. The cyclic di-GMP is synthesized from two molecules of GTP by enzymes known as diguanylate cyclases (DGCs) [13]. The degradation of cyclic di-GMP is catalyzed by enzymes known as phosphodiesterases (PDEs) (**Figure 5**) [13].

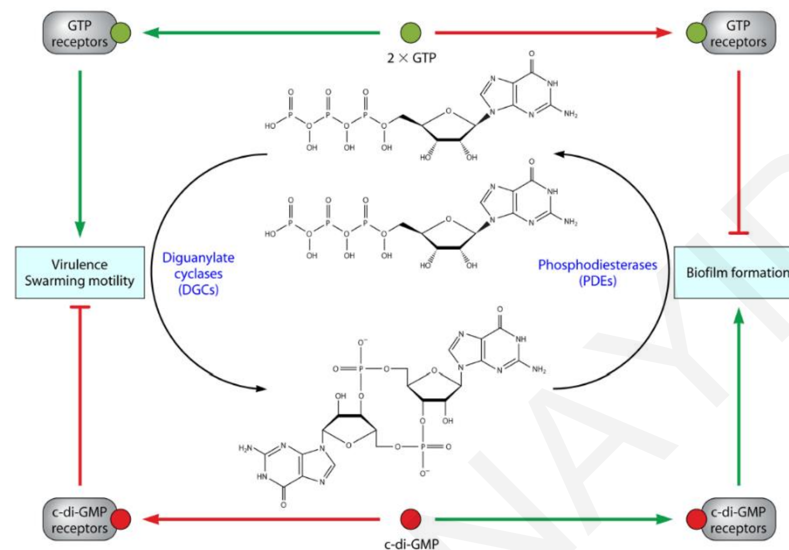

Figure 5. The cyclic di-GMP-mediated regulation of biofilm formation. The levels of cyclic di-GMP are regulated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). Elevated levels of cyclic di-GMP suppress motility and stimulate biofilm formation [13].

1.4 Bacterial metabolism and virulence in *P. aeruginosa*

Metabolism is the sum of the biochemical reactions that occur in a cell or organism. It consists of two interdependent phases: (a) the phase of catabolism by which energy is released through the disintegration of complex organic compounds (such as polysaccharides, lipids, nucleic acids, and proteins) into smaller units (such as monosaccharides, fatty acids, nucleotides, and amino acids, respectively) [50], and (b) the phase of anabolism, which proceeds in the opposite direction with organic compounds being synthesized from simpler compounds [50]. The bacterial growth and replication require carbon and energy that are usually derived from catabolism [50]. For example, in pathogenic bacteria, carbon and energy are usually derived parasitically from the host [50]. This partially occurs by catabolizing macromolecules through the production of virulence factors that can cause oxidative stress and death to the host cells [50]. For this reason, the virulence factor synthesis is significantly regulated by environmental and nutritional signals [50]. This 'nutritional virulence' requires metabolic adaptations [51] in order to maximize the bacterial fitness in a certain growth

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environment [24]. To this end, bacteria evolved a regulatory mechanism known as carbon catabolite repression (CCR) that enables them to increase their fitness by inhibiting the synthesis of enzymes involved in the catabolism of less energetically-favorable carbon sources and giving priority to the catabolism of carbon sources that optimize their growth [52, 53]. Many studies [54-58] were focused on the role of the global metabolic regulator catabolite repression control (Crc) protein in several regulatory processes of *P. aeruginosa* strains. Crc is an RNA-binding protein that acts post-transcriptionally [54, 55]. It was demonstrated that this protein is required for the biofilm formation of *P. aeruginosa* and contributes to the virulence as well as to the antibiotic resistance of this pathogen [56, 57]. Crc binds to a region near to the ribosomal binding site (RBS) of metabolite-transporters and QS-regulatory determinant mRNAs, to regulate their translation [56]. Furthermore, Crc controls the hierarchical assimilation of amino acids in the culture medium leading in metabolism optimization and an increase in the growth rate of *Pseudomonas putida* [58]. In both *P. aeruginosa* and *P. putida*, small non-coding RNAs are regulated by the CbrA/B two-component system (TCS) and antagonize the Crc protein in the presence of less preferred sources [53]. The CbrA/B TCS system regulates multiple pathways that are involved in carbon and nitrogen sources-utilization. CbrA sensor kinase *per se* modulates antibiotic resistance in *P. aeruginosa* [59] and is important for full virulence in a murine acute lung infection model [60]. Moreover, in synergy with its cognate response regulator, CbrB plays a significant role not only in metabolic, but also in other virulence-related processes, including biofilm formation [59, 61].

Virulence factor synthesis and metabolism are interconnected in *P. aeruginosa*. For example, quorum-sensing (QS) has a major effect on *P. aeruginosa* metabolism [62]. During stationary-phase adaptation QS is linked to changes in every major domain of the central metabolism, such as carbohydrate, polyamine and fatty acid/lipid metabolism [62]. Nevertheless, metabolic genes contribute to both growth and virulence. In addition, the enzyme Proline utilization A (PutA), converts proline to glutamate and is also required for virulence of *P. aeruginosa* [63]. Proline is a multifunctional amino acid, which, among others, plays a role in bacterial growth since it is an important source of carbon and nitrogen [63]. A *putA* mutant was attenuated in virulence in a murine acute pneumonia model and more susceptible to oxidative stress, compared to the wild-type strain [63]. Furthermore, the transcriptional regulation of glucose metabolism in *P. aeruginosa* is tightly linked to bacterial virulence and specifically to the regulation of exotoxin A expression [64, 65]. Two regulatory systems, the one-component system PtxS and the two-component system GtrS/GltR, play a central role in this process, directly or through the transcriptional regulator PtxR [64, 65]. Importantly, although homologs of PtxS are found in all *Pseudomonas*, PtxR homologs are present only in human pathogenic

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Pseudomonas species [64]. Importantly, PtxS controls glucose metabolism, in *P. aeruginosa*, *P. fluorescens*, and *P. putida*, while in *P. aeruginosa* PAO1 additionally regulates the expression of the *toxA* gene encoding the exotoxin A [64, 66].

An additional metabolic pathway connected with bacterial virulence is that of pyrimidine biosynthesis, which plays an important role in the pathogenesis and antibiotic resistance of Gram-negative bacteria including *P. aeruginosa* and *E. coli* [67]. The enzymatic activity of the *P. aeruginosa* PyrD can be suppressed by a drug inhibitor, which, reduces the bacterial cytotoxicity, biofilm formation, and the antibiotic resistance [67]. Through the pyrimidine pathway, *P. aeruginosa* utilizes uracil which affects both virulence and biofilm formation [68]. Specifically, uracil biosynthesis affecting mutations, such as *pyrF* that catalyzes the last step in uridine monophosphate synthesis, alter the regulation of the QS systems Las, Rhl, and PQS and also reduce the biofilm formation [68].

Based on recent studies, *P. aeruginosa* is able to utilize exogenous fatty acids to enhance its virulence and survival [69]. Incorporation of several polyunsaturated fatty acids was observed in *P. aeruginosa* phospholipids [69]. Importantly, these exogenous fatty acids increased the bacterial membrane permeability [69]. Several of them also affected the swimming motility and the biofilm formation of *P. aeruginosa* [69].

Conclusively, metabolic processes enable pathogenic bacteria to act more rapidly in new environments, by using available nutrients in a hierarchical manner and consequently facilitate their growth and the production of virulence factors that are required for their prevalence.

1.5 Infection models used in this study

1.5.1 *Drosophila melanogaster* as a model to study host-microbe interactions

The fruit fly, *Drosophila melanogaster* is a great invertebrate model organism that reflects some aspects of the mammalian pathogenesis of infection [70, 71]. Its short life cycle, small size (~2mm in length) and ease of rearing allow the production of up to ~50 adult progeny per female fly within 2 weeks, thus facilitates the *in vivo* large-scale screening of bacterial mutants. Many human bacterial, fungal and viral infections can be studied in *Drosophila* [72]. Notwithstanding the lack of adaptive immunity as we know it in mammals, *Drosophila* has similar innate immunity, disease-related signaling pathways and cell types to those of mammals. Thus, it is a good model for studying the pathogenicity of microbial infections, including those caused by *P. aeruginosa* [70, 71, 73].

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The defenses of *Drosophila* upon microbial infection include both humoral and cellular immune responses that limit the microbial proliferation [74]. The humoral response involves the secretion of antimicrobial peptides (AMPs) into the fly hemolymph (insect blood) [75]. The AMPs are rapidly produced and released by the fat body in response to infection, while under normal conditions are constitutively expressed in some barrier tissues at low levels [76]. The fat body is analogous to the vertebrate liver and adipose tissue [75]. During the humoral immunity, the induction of AMPs in the fat body relies on the activation of two major signaling pathways, the Toll and Imd pathways and the further activation of the NF-κB signaling [77]. The Toll pathway is mainly activated by fungi and Gram-positive bacteria, whereas the Imd pathway is usually activated in response to the infection caused by Gram-negative bacteria [77]. On the other hand, the cellular immune responses include the recruitment of different types of hemocytes (insect blood cells), with several and distinct functions, at the site of the infection. These functions include: (a) phagocytosis of small intruders such as bacteria, (b) encapsulation of larger intruders and (c) nodule formation in the case of a large number of smaller intruders [75].

There are three most common methods to infect *Drosophila* with *P. aeruginosa*: [2, 73, 78] (a) the feeding method involves mixing of bacteria with the fly food, which causes intestinal colonization and flies lethality within a few days; (b) the thoracic or abdominal needle pricking infection, that is, an injury using a tungsten needle dipped into a bacterial suspension. Accordingly, bacteria are introduced locally at the wound site and later on spread systemically killing the flies within 2-4 days; and (c) the injector pumping appears similar to the pricking method however, is a method of systemic infection and involves the injection of a controlled dose of bacteria directly into the fly hemocoel with a thin glass capillary tip [78]. The latter two methods have been used to screen *D. melanogaster* for virulence-related mutants of the *P. aeruginosa* strain PA14, for example, the virulence-attenuating factor *hudA* [79] and the hypothetical methyltransferase KerV, that is conserved among Proteobacteria [80]. Moreover, NF-κB and JNK signaling pathways are important for flies to resist *P. aeruginosa* infection [81], although highly virulent *P. aeruginosa* escapes host defenses by suppressing or evading the induction by these pathways that normally induce antimicrobial peptides systemically and muscle genes at the wound site [82,83]. In addition, transgenic expression of the human lactonase paraoxonase-1 (PON1) in flies protects them from *P. aeruginosa* wound infection by interfering with the bacterial quorum-sensing [84]. Thus, human innate immunity factors, such as PON1, can be introduced and studied in *Drosophila* [85]. Using the oral infection model, which recapitulates intestinal colonization and systemic dissemination of *P. aeruginosa,* new aspects of bacterial quorum-sensing and intestinal pathology have been

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revealed. For example, the *Drosophila* chromatin remodeling factor chromo helicase domain protein 1 (CHD1) contributes to fly intestinal resistance to *P. aeruginosa* infection [86] and the quorum-sensing factor *rhlR* contributes to circumvent the fly cellular immune response when bacteria escape the intestine and spread systemically [87]. Both RhlIR and LasIR quorum-sensing systems are required for full virulence in orally infected flies [88]. In addition, intestinal *P. aeruginosa* senses Gram-positive bacterial peptidoglycan to enhance its quorum-sensing-mediated virulence [89]. Strikingly, intestinal *P. aeruginosa* and the quorum-sensing virulence factor pyocyanin induce intestinal stem cell-mediated regeneration, which facilitates tumorigenesis in the presence of oncogenes or in the absence of tumor suppressor genes [90]. Moreover, the activation of the JNK innate immune signaling pathway, in the adult *Drosophila* hindgut cells during *P. aeruginosa* infection, synergizes with $Ras1^{V12}$ oncogene expression to induce enterocyte invasion and dissemination to distant sites [91, 92].

1.5.2 Acute lung infection mouse models

Acute microbial lung infection that mimics the human acute bacterial pneumonia, can occur in mice upon exposure to infectious aerosols or directly by intranasal or intratracheal instillation [93]. Intranasal infection allows the spreading of the bacteria from the upper airways to the intestine and the lower airways [94]. In contrast, intratracheal instillation delivers much more bacteria into the distal bronchi [94]. *P. aeruginosa* acute lung infection murine models can be used to gain insights about the immune responses and the lung function of the infected animals [95]. In mice infected intratracheally with *P. aeruginosa* there is a correlation between increased expression of interleukin-6 (IL-6), edema formation and decreased lung function [95]. In addition, the pro-inflammatory cytokine interleukin-17 (IL-17) facilitates the recruitment of neutrophils in the infected lung areas of infected mice [96]. On the other hand, immunosenescence leads to the impaired neutrophil response, as observed in aged versus young mice subjected to intratracheal infection [97]. Septic mice infected intratracheally induce interleukin-27 (IL-27), which in turn induces immunosuppression [98]. Towards the standardization of new therapeutic approaches against human *P. aeruginosa* lung infections, Lawrenz *et al.* proposed recently a leukopenic (cyclophosphamide-treated) mouse model of lung intratracheal instillation for therapeutic testing of novel drugs against multidrug-resistant strains [99].

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CHAPTER 2

SIGNIFFICANCE, HYPOTHESIS & SPECIFIC AIMS

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2.1 Significance

P. aeruginosa has long been recognized as a life-threatening human opportunistic pathogen causing acute wound infections and persistent lung infections able to develop resistance to multiple antibiotics [100]. This pathogen can also infect a wide range of hosts including animals, insects, and plants using a repertoire of virulence factors, thus investigating *P. aeruginosa* infection using model hosts is useful and common practice in virulence assessment [78]. The current worldwide spread of antibiotic resistance demands novel approaches for anti-infective therapy. Novel bacterial targets must meet specific criteria in order to be ideal for drug development [101]. For example, it is important for novel targets to exist in most of the pathogenic strains within a specific species [101]. In addition, they must be highly conserved at the sequence level in various strains [101]. On the other hand, potential targets that have human homologs must be excluded, to avoid toxicity [101]. Bacterial components essential for growth and survival, have a tendency to be considered as ideal targets for identifying antibacterial compounds [101]. However, antibiotics that inhibit bacterial growth are more likely to cause the development and spread of antibiotic resistance [102-104].

Our long-term goal was to identify virulence-essential metabolic genes that could serve as novel targets for drug development against *P. aeruginosa*. The high adaptability and regulatory signaling network complexity of this bacterium, enable its virulence in many hosts, including humans [13]. Metabolic genes have been found on pathogenicity islands, which may contribute to the capacity of the bacteria to colonize new host niches [105]. Moreover, pathogenic bacteria usually undergo mutational adaptations depending on the nutrient availability in a certain environment [105]. For example, mutations in virulence factor genes upon high nutrient availability may result in a growth advantage where although these bacteria will be less virulent, they will be better adapted in that environment [105]. Accordingly, in chronic human infections, pathogens losing virulence factors may have a metabolic gain [105]. However, the extent of the contribution of the metabolic genes and pathways in virulence is not clear. While the effect of nutrient availability and bacterial replication in colonization was first shown a century ago, the studies of bacterial metabolism and bacterial virulence progressed independently hence our knowledge on metabolic genes linked to virulence remains fragmented. To fill this gap in our knowledge, we assessed the impact of all metabolic genes of *P. aeruginosa* for their role in virulence.

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2.2 Hypothesis and Specific Aims

2.2.1 Deciphering the link between *P. aeruginosa* metabolism and virulence

Bacterial metabolism is important for both growth and survival. In this study, we focused on bacterial metabolic genes that are important for full virulence, because virulence factor production is crucial for bacterial survival, especially in new hostile environments.

Our **hypothesis** was that a great percentage of *P. aeruginosa* metabolic genes while dispensable for growth are essential for the full virulence of this pathogen. Previous studies have shown the contribution of specific metabolic genes or pathways in the virulence of *P. aeruginosa* however, a large-scale study, that describes the extent to which metabolic genes of *P. aeruginosa* contributes to its virulence, did not exist. Furthermore, the identification of virulence-related metabolic genes could be a novel approach for identifying new drug targets that could mitigate the development and spread of antibiotic resistance.

The following **specific aims** were implemented to examine our hypothesis:

(1) Identify *P. aeruginosa* metabolic genes that are essential for full virulence in *Drosophila melanogaster* infections.

(2) Assess the ability of the selected mutants to grow efficiently in culture and in the host. Virulence factor production by metabolic mutants exhibiting growth retardation phenotypes, cannot be assessed in the host because they may be primarily essential for growth rather than for virulence.

(3) Validate the attenuation in virulence of representative *P. aeruginosa* metabolic mutants in an acute murine lung infection model.

(4) Assess the virulence factor production in representative virulence-related *P. aeruginosa* metabolic mutants.

(5) Further investigation of selected virulence-related metabolic mutants for virulence factor production and gene complementation analysis.

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CHAPTER 3

METHODOLOGY

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3.1 Bacterial Strains and Growth Conditions

3.1.1 Transposon Insertion Mutant Library

The catalog of the metabolic genes of *P. aeruginosa* strain PA14 was taken by the KEGG Database. The metabolic mutants were picked from the 96-well plates, of the publicly available PA14 Transposon Insertion Mutant Library (the PA14NR Set) (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi). This is a nonredundant library of PA14 transposon mutants in which nonessential PA14 genes are represented by a single transposon insertion chosen from a comprehensive library of insertion mutants [106]. The parental library of these mutants was generated by using MAR2xT7, a transposon compatible with transposon-site hybridization [106].

3.1.2 Growth Conditions

The bacterial strains are stored at -80°C. Before each experiment, fresh lysogeny broth (LB) agar plates were made where the bacterial strains were grown for 16 hours at 37°C. The plates were kept at 4°C for a maximum of 10 days. Prior to each experiment, a single colony was selected from the plate of each strain and bacteria were grown overnight in LB broth liquid cultures (3 ml each) for 16-18 hours at 37°C with shaking at 200rpm. The next day new liquid cultures were placed by inoculating, 1:100 dilution of the over-night culture, into fresh LB broth.

3.2 Fly Strains and Growth Conditions

For the infection assays, we used wild-type Oregon R flies 3-7 days old grown in incubators that have a standard temperature (25°C) and humidity (65%).

3.3 Infection Assays

3.3.1 Pricking Assay

Male Oregon R flies were pricked in the thoracic cuticular epithelium with a tungsten needle dipped in a bacteria suspension, as previously described [78]. The infection mix consisted of 980 μl ddH_2O, 10 μl 1M $MgSO_4$ and 10 μl of bacteria OD_{600nm}: 3.0. Vials were transferred at 25°C and survival was measured every day. For each mutant, we had 2 vials with 20 flies (40 flies in total) in order to make a better statistical analysis. About 10-14 mutants were assessed each time.

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3.3.2 Feeding Assay

For the feeding assay, female Oregon R flies 3-7 days old were starved for 5-6 hours and then were transferred in vials containing the following feeding mix on cotton: 0.5 ml of bacteria OD_{600nm}: 3.0, 1 ml 20% sucrose and 3.5 ml ddH_2O. Vials were transferred at 25°C and survival was measured every day. For each mutant, we had 3 vials with 10 flies (30 flies in total) in order to make a better statistical analysis.

3.4 Statistical analysis

3.4.1 Statistical analysis of the screen results.

In the first phase of the screen 553 metabolic mutants (482 metabolic genes) of PA14, were tested for their virulence by using *Drosophila melanogaster* as an infection model in two independent assays: the pricking assay which is a wound infection and the feeding assay which is an oral infection. The two assays were performed in parallel and a set of 10-14 mutants were screened each time.

At the end of the pricking assay, we calculated the normalized value of the percentage of flies' survival for each one of the 553 metabolic mutants. First, we calculated the average between the counts of flies survival of the two vials which we had for each metabolic mutant and then we calculated the average of the flies survival for all the mutants of a certain experiment (for example in the day 1 we assessed 10-14 mutants). Then we divided the average of the flies' survival for each mutant with that value. For example, the *Normalized value of the % survival of the mutant X of the day 1 = Average of the % survival of the mutant X of the day 1 / Average of the % survival of the all mutants of the day 1.* Next, we found the standard deviation using the normalized values of the % flies' survival of all the days. Finally, in order to find the Z-score value for each mutant, we used this equation: *Z-score value= (Normalized value of the % survival/Standard deviation) – Average of (Normalized value of the % survival/Standard deviation).* We used the Z-score values of all the mutants in order to create the final graph and based on it we selected the most attenuated mutants for verification. Moreover, we made Kaplan-Meier analysis (log-rank test) for the remaining mutants and from the p-values, we selected the mutants that were the most attenuated in virulence ($p<0.05$).

For the feeding assay, using the counts of the survival of the flies for each mutant which were given by the 3 vials, we made a graph of survival as a function of the time in hours. Then from that graph, we found the Lethal Time 50% (LT-50) for each mutant, which is the time in which 50% of the flies are dead. Then we found the Normalized value of the LT-50. For example, the *Normalized value of the LT-50 of the mutant X of the day 1 = LT-50 for the*

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mutant X of the day 1/Average of all the LT-50s of all the mutants of the day 1. Using the Normalized values of the LT-50s of the flies for the mutants of all the days we then calculated the standard deviation. Finally, in order to calculate the Z-score value for each mutant, we used this equation: *Z-Score Value = (Normalized Value of LT-50/Standard Deviation) – Average of (Normalized Value of LT-50/Standard Deviation).* We used the Z-score values of all the mutants in order to create the final graph and based on it we selected the most attenuated mutants for verification. Moreover, we made Kaplan-Meier analysis (log-rank test) for the remaining mutants and from the p-values, we selected the mutants that were the most attenuated in virulence ($p<0.05$).

The selected metabolic mutants were verified by Kaplan-Meier analysis (log-rank test) ($p<0.05$) after wound (pricking assay) or oral infection (feeding assay) of the flies. The mutants that were verified by this analysis were selected for further experiments.

3.4.2 Statistical analysis of the results of the screen of *P. aeruginosa* non-metabolic genes.

We had also assessed 100 non-metabolic mutants (randomly selected from the Transposon Insertion Mutant Library), for virulence using both pricking and feeding assay. The attenuated non-metabolic mutants were then selected by Z-score and Kaplan-Meier analysis and were retested for verification.

3.5 Growth Assessment Assays

3.5.1 *In vitro* assays for the determination of the growth ability of the selected metabolic and non-metabolic *P. aeruginosa* mutants

3.5.1.1 Growth in Glucose Minimal Media

For this assay 500 μl of bacteria that were grown in 3 ml LB O/N cultures were centrifuged at 8000 rpm for 2 min, the supernatant was removed, and the pellet was diluted in minimal medium. From this mix 1:100 was left to grow in minimal medium on a culture rotator at 37°C. For each mutant we had duplicates and three time points of the optical density OD_{600nm} were taken.

Glucose minimal medium protocol for 50 ml: 10 ml 5xM9, 100 μl 1 M $MgSO_4$, 1 ml 20% glucose, 39 ml ddH_2O.

M9 components: 33.9g/L Na2HPO4, 15g/L KH2PO4, 5g/L NH4Cl, 2.5g/L NaCl. We made a 5x concentrated stock solution by stirring to suspend 56.4g powder in 1L water and we used it after autoclave sterilization.

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3.5.1.2 Growth in Glucose Minimal Media with 5% Fly Extract

For this assay, we followed the same steps as in paragraph **3.5.1.1**, with the only difference that we had 5% fly extract in the glucose minimal medium.

The fly extract was produced using the following protocol:

First, we collected adult flies into 50 ml plastic disposable centrifuge tubes. We put them in the freezer for at least 45 minutes. The flies can be used once they are quiescent or stored in the freezer for future use. The genotype of the flies is unimportant; however, we used the same wild-type strain as in our screen (Oregon R flies). For every 100 ml of final medium approximately 300 flies (about 0.35 g) are needed. The following steps are needed in order to produce the extract: First, we weigh the flies by transferring them to a tared tube. Then we transfer the flies plus 6.8 ml medium per gram of flies into a homogenizer (it must stay cold since tyrosinase is activated during homogenization and melanization can ruin the extract). Spin the tube at 1500 X g at 4°C for 15 min. Collect the supernatant into fresh tubes and incubate it at 60°C for 5 min in order to inactivate tyrosinase. Spin at 1500 X g at 4°C for 90 min. Collect the supernatant; this is the fly extract. Filter-sterilize the extract through a 0.22 μm filter. The fly extract can be stored at -20°C. For our assays, we added fly extract to glucose minimal medium to a final concentration of 5%.

3.5.2 Colonization Assays

3.5.2.1 Wound Colonization Assay in flies

The colonization ability of the pricking assay-selected metabolic mutants was examined by counting CFUs from ground tissue of flies previously injected with an amount of 10^2 bacteria. For each mutant and for the wild-type strain, 20-25 flies were injected dorsoventrally as previously described [78] and then transferred into vials with fresh food (at 25°C). The next day (18-24 hours after), plates were cultured with a solution that contained ground tissue of the flies in order to count CFUs and observe the colonization ability of each mutant, compared to the wild-type strain.

3.5.2.2 Intestinal Colonization Assay in flies

The colonization ability of the feeding assay-selected metabolic mutants was examined, using Oregon R flies 3-7 days old, previously starved for 5-6 hours and then fed with bacteria OD_{600nm}: 3.0 for one day. The flies were next transferred into 50 ml Eppendorf with 12 holes (1.2 mm in diameter) on the lid. Flies were able to reach food (Whatman filter paper disc soaked with 200 μl of 4% sucrose and 10% LB set on the lid and covered with parafilm) only by the holes of the lid. Flies were transferred in clean 50 ml Eppendorf every day for 3

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days to avoid contamination. On the third day, plates were cultured with a solution that contained ground tissue of the flies in order to count CFUs and observe the colonization ability of each mutant, compared to the wild-type strain.

3.5.2.3 CFUs from flies

A set of 3 flies for each mutant, was bathed in ethanol and then ground by hand in 1.5 ml Eppendorf tubes having 100 μl LB. After the grinding, an additional amount of 900 μl LB was added in each Eppendorf tube. Finally, 10μl of each Eppendorf tube was plated in LB plates and the plates were incubated overnight at 37°C. CFUs were counted the next morning. For each mutant and for the wild-type strain we had 3 replicates. For each mutant, we calculated the average and the standard deviation of the CFUs of the 3 plates, in order to create the graphs and make a correlation with the wild-type strain.

3.6 Intranasal Mice Lung Infection

The intranasal infection achieves the spreading of the bacteria from the upper airways to the intestine and low airways thus mimics the pathology seen in acute bacterial pneumonia [107,108]. PA14 wild-type and mutant strains were grown in LB liquid cultures overnight. Cultures are then diluted 1:100 and were grown over the day until to reach the optical density OD_{600nm}: 3.0 ($3.0x10^9$ CFU/ml). Bacteria were pelleted and washed twice in sterile saline (0.9 %) and a required dilution was done in order to reach the desired infectious dose of $2x10^7$ CFU/mice. Mice were intranasally infected, under very short and light anesthesia, as previously described [109,110], by placing 10μl of a bacterial suspension in each nostril (20μl in total). Mortality counts were taken every day for 7 days. CD-1 female mice 6 weeks old, were used in this study.

3.6.1 Ethics Statement

Animal protocols have been approved by the Cyprus Veterinary Service inspectors under the license number CY/EXP/PR.L6/2018 towards the Laboratory of Prof. Apidianakis at the University of Cyprus. The veterinary services act under the auspices of the Ministry of Agriculture in Cyprus and the project number CY.EXP101. These national services abide by the National Law for Animal Welfare of 1994 and 2013 and the Law for experiments with animals of 2013 and 2017.

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3.7 Quantitative reverse transcriptase real-time PCR

RNA isolation was performed from log-phase cells grown to an OD_{600nm} of 1.0 or 2.0 using the QIAzol Lysis Reagent. Briefly, 500 µl of bacteria OD_{600nm} of 1.0 or 2.0 (~10^9) have spun down at 8000 rpm for 3 minutes. The pellet was dissolved by adding 500 µl of QIAzol and by pipetting up and down at 60°C for 10 minutes. Next, 100 µl $CHCl_3$ was added and tubes were inverted for 15 seconds. After a 5 minutes incubation at room temperature, the supernatant (300 µl) was selected in a new tube, by a full-speed centrifugation at 4°C for 15 minutes and then mixed with 300 µl iso-propanol by inverting the tubes. Tubes were then let on the bench for 5 minutes and after a step of full-speed centrifugation at 4°C for 10 minutes, the pellets washed out with 500 µl of 70% EtOH. Finally, after centrifugation at 4°C for 3 minutes and air drying, pellets resuspended in 20-50 µl RNase free H_2O by pipetting and stored at -80°C. The bacterial RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT reagent Kit (Perfect Real Time) (Takara: RR037A) according to the manufacturer's instructions. Real-time PCR was performed, using a Bio-Rad CFX1000 thermal cycler and Bio-Rad CFX96 real-time imager with primer pairs listed below and iQ SYBR green supermix (Bio-Rad). Results are from two independent experiments performed in biological triplicates. All samples were normalized to the expression of the housekeeping genes *rplU* and *clpX* via the Pfaffl method [111].

#	Target ID	Target primers sequence (5'- 3')
1	*rplU*	F: ATGGCGAAGACGTGAAAATC
		R: GAACTTGATGATGCGGACCT
2	*clpX*	F: GTTCGGTCTTATCCCCGAGT
		R: AACAGCTTGGCGTACTGCTT
3	*exsA*	F: TTGGCCGAAAGCAGATAACG
		R: TACGCCCTCTTCCTTGTTTACC
4	*exsC*	F: CACCGTTTCGATCTGCATTTCG
		R: CGAGAATCTGCGCATACAACTG
5	*exoT*	F: GCCGAGATCAAGCAGATGAT
		R: TTCGCCAGTCTCTCCTCTGT
6	*exoU*	F: CCTTTTGGCCTCAGGTATGA
		R: CTCGCTGCTAATGTGTTGGA
7	*pa1L*	F: GGTTGCACCCAATAATGTCC
		R: CCAATATTGACGCTGAACGA
8	*pilA*	F: CAGAGGCGACTGGTGAAATC
		R: AGGGTAGAGTCAGCCGGAAT
9	*clpV1*	F: TGAACAGCCTGGCCTACAAG
		R: ACCAGCTCGACATAGGGATTG
10	*hcp1*	F: AGTCCAAGGACAAGACTCACG

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		R: TGTACTTGGTGAACGACAGGTC
11	*vgrG1*	F: GCGCTTCTTTCACGGTATCG
		R: ACGCTCTGGTTCTGGAAGATG
	"F" and "R" indicate the direction (forward or reverse) of the primer related to the target gene sequences.	

3.8 Motility Assays

3.8.1 Swarming motility

Swarming motility was performed using Petri dishes each containing exactly 20 ml of medium consisted of 5 g/l Bacto-agar (Difco), 8 g/l Nutrient Broth (Difco) and 5 g/l Dextrose. Bacterial cultures, with the strains of interest, were grown overnight in LB medium and 2 μl from each culture was added at the center of a swarming plate. The plates stayed open until the droplet is fully absorbed by the agar and then incubated for 24 hours at 37°C. The diameter of the swarming zone on the plate was measured, and the photos were taken.

3.8.2 Swimming motility

To examine the swimming motility, the indicative strains were grown in LB cultures until to reach the optical density OD_{600nm}: 3.0 and then inoculated on Petri dishes containing 0.3% Bacto agar (Difco), 1.0% Tryptone and 0.5% NaCl, by pricking the agar with a sterile toothpick dipped in the bacterial culture of each strain. The plates were then incubated at 37°C for 16-24 hours. The diameter of the swimming zone on the plate was measured, and the photos were taken.

3.8.3 Twitching motility

Twitching motility was performed using Petri dishes containing 1.0% Bacto agar and 20 g/L LB broth. After the agar was solidified, the indicated strains were stabbed at the bottom of the plates with a sterile toothpick. The plates were incubated at 37°C for 48 hours. The ability of the bacteria to adhere and form biofilms was examined by removing the agar, washing the unattached cells with water and staining the attached cells with crystal violet (1%). The stain solution was removed by carefully washing the plates with water. The diameter of the twitching zone on the plate was measured, and the photos were taken.

3.9 Quantification of Biofilm formation

The biofilm formation of the wild-type *P. aeruginosa* or mutant strains was grown, measured and quantified as previously described by George A. O'Toole [112]. Briefly, overnight cultures of the indicated strains were diluted 1:100 into fresh M63 minimal medium supplemented with magnesium sulfate and arginine. Next, 100 μl of each dilution were added per well in

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a 96 well dish and this was transferred at 37°C for about 24 hours. We used 3-5 replicate wells for each strain. After the incubation, the unattached cells were carefully removed by washing the 96 well dishes twice with water. The attached cells were then stained by adding 125 μl of 0.1% of crystal violet solution, in each well of the microtiter plate. The plate was incubated at room temperature for 15 min and then washed with water 3-4 times. Finally, the plate was let to dry overnight, and wells were then photographed. To quantify the biofilm, 125 μl of acetic acid in the water, was added to each well of the microtiter plate and this was then incubated at room temperature for 15 min. The absorbance at 590 nm was quantified in Nanodrop Spectrophotometer, by using 30% acetic acid as a blank.

3.10 Plasmid Complementation

3.10.1 Colony PCR for the *surE* gene and DNA Purification

The colony PCR technique was used to amplify and isolate the *surE* gene from the wild-type PA14 strain. A single bacterial colony was picked up from an LB plate using a sterile tip and placed into a sterile Eppendorf tube which contained 50 μl RNase-Free water. Next, the tube was heated at 95°C for 6 minutes and then placed on ice for 10 minutes. A PCR reaction was performed in a solution with a final volume of 50 μl, using the KAPA HiFi HotStart DNA Polymerase PCR Kit (KK2501). The half of this amount (25 μl) was used in gel electrophoresis to check whether the PCR worked, while the rest amount of the DNA was purified, in order to be used at the next steps, using the DNA purification protocol of the Wizard® SV Gel and PCR Clean-Up System of Promega as per manufacturer's instructions.

[μl] [25 μl]	**PCR components**		**PCR conditions**		**[°C]**
13.25	RNase free water		1 cycle	5 min	95
5.0	5X KAPA HiFi Fidelity Buffer		25 cycles	20 sec	98
0.75	10 mM dNTP Mix			30 sec	60
0.75	10 μM FW Primer			60 sec	72
0.75	10 μM RV Primer		1 cycle	5 min	72
1.5	$MgCl_2$ (25 mM)			∞	4
2.5	Template DNA				
0.5	1 U/μl KAPA HiFi				

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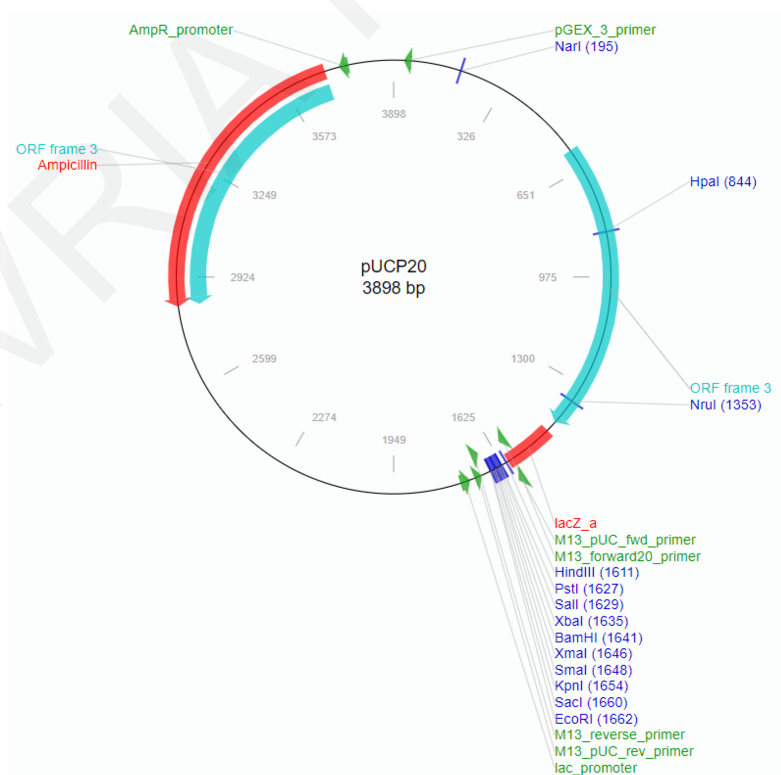
Plasmid	Restriction Site	Cloning Primers for the *surE* gene
pUCP20	XbaI	GGTCTAGAGTGGTGCGTGAAATCCTCGA
	HindIII	GGGAAGCTTTCACGTCAGTCCTCCCAGC
pDN19	HindIII	GGGAAGCTTGTGGTGCGTGAAATCCTCGA
	XbaI	GGTCTAGATCACGTCAGTCCTCCCAGC

3.10.2 Cloning Vectors

The *surE* gene is in the middle of a predicted operon thus, it was easier to use an expression plasmid with a constitutive promoter. Moreover, we didn't know if the overexpression of *surE* could be toxic or detrimental to the bacterium. For this reason, we used two compatible plasmids pUCP20 and pDN19, with high or low copy numbers respectively, and different strengths of their constitutive promoters. Both vectors have a lac promoter (promoter from lac operon) which is primarily used for general expression and can be induced by IPTG or lactose. The pDN19 vector has also a T7 promoter (promoter from T7 bacteriophage) which is constitutive but requires T7 RNA polymerase. More information about these promoters can be found at *https://blog.addgene.org/plasmids-101-the-promoter-region*.

The plasmids maps were taken by *https://www.addgene.org* and are shown below.

Vector pUCP20



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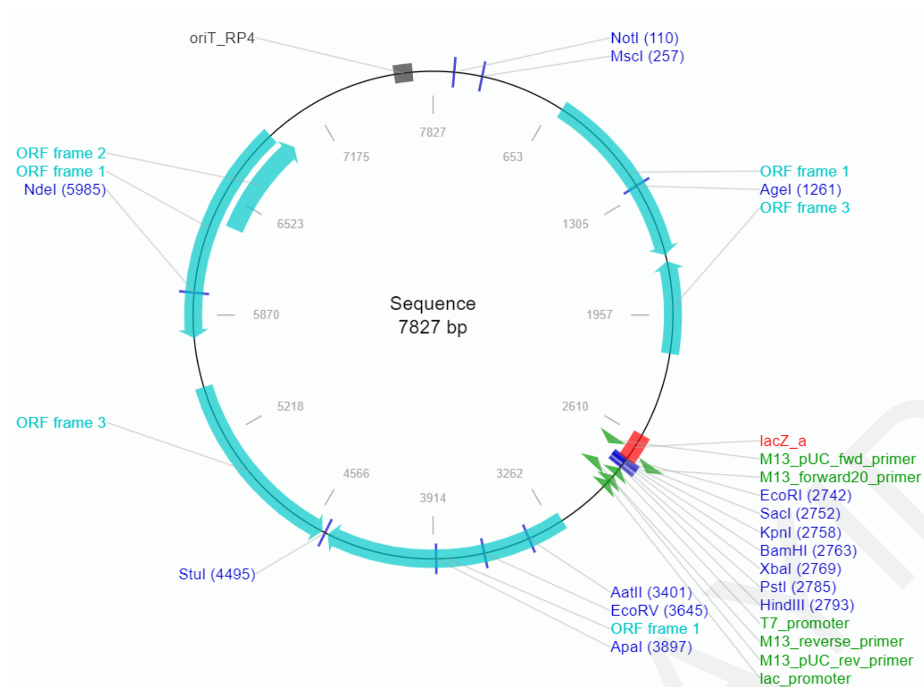
Vector pDN19



3.10.3 Plasmid Isolation

The isolation of the pUCP20 and the pDN19 plasmid from the *E. coli* DH5α cells was performed using the Plasmid DNA Purification Kit of Macherey-Nagel as per manufacturer's instructions. Specifically, for the isolation of pUCP20, we used the protocol of isolation of high-copy plasmid DNA from *E. coli* while, for the isolation of pDN19, the protocol of isolation of low-copy plasmids.

3.10.4 Double Digestion and Ligation

The cloning vectors pUCP20 and pDN19 as well the *surE* DNA with the compatible restriction sites for each one of the two vectors, were digested with the XbaI and HindIII restriction enzymes of Takara using the following reaction:

Double Digestion Compounds	Reaction [50 μl]		Conditions	[°C]
DNA	~ 2.5 μg		4 hours	37
10X Digestion Buffer	5 μl		15 min	70
1st enzyme (XbaI)	3 μl			
2nd enzyme (HindIII)	3 μl			
ddH_2O	Rest of volume			

The ligation was performed using the T4 DNA Ligase and the 5X DNA Ligase Reaction Buffer of Invitrogen (Cat. No. 15224-017) as follows:

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Ligation Conditions for Cohesive Ends - Reaction [20 µl]	
5X Ligase Reaction Buffer	4 µl
Insert: Vector Molar Ratio	3:1
Vector Ends	3-30 fmol
Insert Ends	9-90 fmol
Total DNA	0.01-0.1 µg
T4 DNA Ligase	0.1 unit
Autoclaved distilled water	Rest of volume
Temperature	25 °C
Time	1 h

3.10.5 Preparation of Calcium Competent *Escherichia coli* DH5α Cells

For this procedure, we used the protocol for calcium competent cells of the Krantz Lab that is available online (http://mcb.berkeley.edu/labs/krantz/protocols/calcium_comp_cells.pdf), with a few modifications. Specifically, an overnight culture of *E. coli* DH5α was inoculated into LB medium and incubated at 37°C for about 18-24 hours with vigorous shaking. The next day the overnight culture was diluted 1:100 in fresh LB (300 ml overday culture) and grown in 37°C shaker until to reach the optical density OD_{600nm}: 0,35-0,40. When the culture reached that optical density, immediately chilled on ice for 20-30 minutes. At the same time centrifuge vials were put on ice because, the cells but also any bottles or solutions that are in contact with them, should be pre-chilled at 4°C. The 300 ml culture was split into 6 parts by pouring about 50 ml into ice-cold sterile centrifuge tubes. The cell pellets were collected by centrifugation at 6000 rpm for 15 minutes at 4°C. The supernatant was discarded, and each pellet was resuspended in 25 ml of ice-cold 100 mM $MgCl_2$. The cells were next collected by centrifugation at 6000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the cell pellets were resuspended in 25 ml of ice-cold 100 mM $CaCl_2$. This suspension was kept on ice for about 20 minutes and then the cell pellets were collected by centrifugation at 5000 rpm for 15 minutes at 4°C. The supernatant was discarded, and each pellet was resuspended in 5 ml of ice-cold 85 mM $CaCl_2$ with 15% glycerol. Next, all of them were transferred to a 50 ml tube. Finally, after another step of centrifugation at 5000 rpm for 15 minutes at 4°C, the supernatant was discarded, and the pellet was resuspended in 3 ml of ice-cold 85mM $CaCl_2$ with 15% glycerol. The competent cells were dispensed into aliquots of 300 µl and stored at -80°C.

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3.10.6 Heat-Shock Transformation

To proceed with the transformation of the calcium competent *E. coli* DH5α cells, 2 tubes with 100 μl of those cells were transferred on ice and 1 μl of plasmid solution (pUCP20-*surE* or pDN19-*surE*) was added to each one of them, to have a final concentration of 1μg/μl. The next step was incubation of the tubes on ice for 30 minutes, followed by a heat-shock at 42°C for 2 minutes and again transfer to ice. After this step, the cells were transferred to tubes contained 900 μl of sterile LB and incubated at 37°C for about 30-45 minutes with vigorous shaking. Finally, 100 μl of each culture was plated onto X-gal/IPTG plates contained the appropriate antibiotics and incubated at 37°C for 18 hours. The next day, the white colonies from each plate, which contained the plasmid of interest, were collected and overnight cultures were prepared in order to stock them for future use.

3.10.7 Preparation of *P. aeruginosa* Competent Cells and Plasmid Transformation

The preparation of *P. aeruginosa* competent cells, as well as the plasmid transformation, was performed as previously described [113].

For the preparation of *P. aeruginosa* competent cells, 1 ml of an overnight culture was first transferred into a pre-chilled microcentrifuge tube and centrifuged at 13000x g for 30 seconds at room temperature. The supernatant was decanted, and cells were suspended in 1 ml of cold 0.1 M $MgCl_2$. Centrifugation was repeated as previous at 13000x g for 30 seconds at room temperature and cells resuspended in 1 ml of cold TG salts solution (75 mM $CaCl_2$, 6 mM $MgCl_2$, 15 % glycerol). The tube was incubated on ice for 10 minutes and then centrifuged again as above. Finally, cells resuspended in 200 μl cold TG salts.

For the transformation, 2-5 μl (100-400 ng) of plasmid DNA, was added to 100 μl of *P. aeruginosa* PA14 competent cells which were sitting in a tube on ice and incubated there for 15 minutes. After a heat-shock step at 37°C for 2-5 minutes, 500 μl of LB was added to the cells, followed by incubation at 37°C for 1 hour. Finally, 200 μl of each culture was plated onto LB plates containing the appropriate antibiotics. We also performed a transformation of empty pUCP20 or pDN19 vectors, into wild-type PA14 cells (control samples).

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CHAPTER 4

RESULTS

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4. Deciphering the link between *P. aeruginosa* metabolism and virulence

4.1 Assessing the percentage of the metabolic genes of *P. aeruginosa* strain PA14 contributing to virulence

To functionally assess the contribution of the metabolic genes of *P. aeruginosa* to virulence, we took advantage of the PA14 unigene Transposon Insertion Mutant Library. The PA14 strain is very well annotated and one of the highly pathogenic strains we used in this study. Its genome comprises approximately 6,537,648 nucleotides and 5,977 genes [114, 115], 13.3% of which are metabolic genes (794 metabolic genes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database). Using the PA14 mutant library, we investigated all available and viable metabolic gene mutants for virulence in *Drosophila*. We assessed 648 mutants corresponding to 482 metabolic (553 mutants) and 94 randomly selected non-metabolic (95 mutants) genes, in the pricking (wound) and feeding (oral) infection fly assays. For the screen, wild-type female flies 3-7 days old were orally infected with PA14 strains diluted in a sucrose solution. In addition, male wild-type flies, grown together with the female flies, were wound infected with a needle dipped in a bacterial solution containing one PA14 strain at a time. In both cases, the survival of flies was counted for 4-7 days. The time of the 50% fly death (LT50%) was assessed for each mutant and condition and a Z-score analysis was used to select those with a standard deviation of more than +1 (**Figure 6 A, B, C, D**). Selected mutants were retested for virulence and the final number of the attenuated mutants was finalized by calculating the p-value, using Kaplan-Meier survival analysis with a log-rank test. Considering both assays, 16.2% (78/482) of the metabolic and 8.5% (8/94) of the non-metabolic *P. aeruginosa* genes, were found virulence-defective in flies. Of note, metabolic gene mutants might be impaired in growth e.g. may exhibit auxotrophy rather than being directly involved in virulence factor production.

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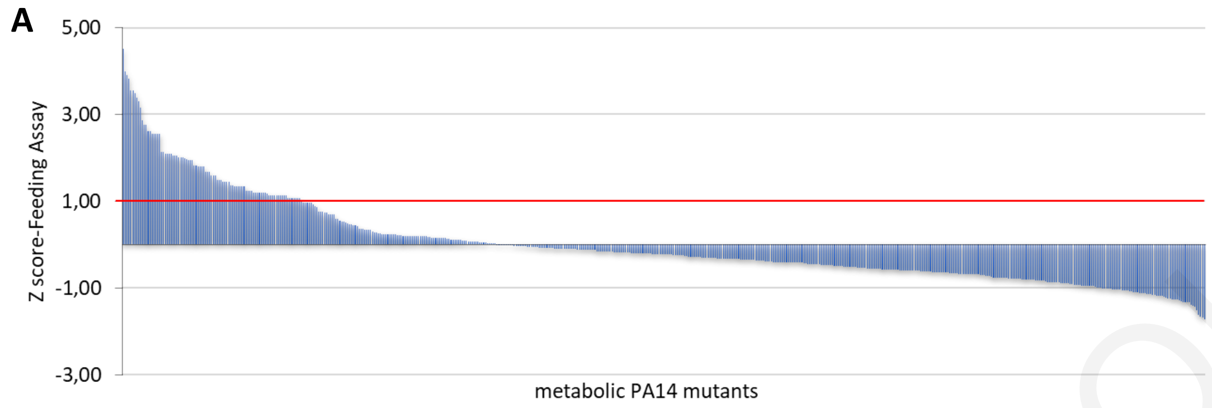

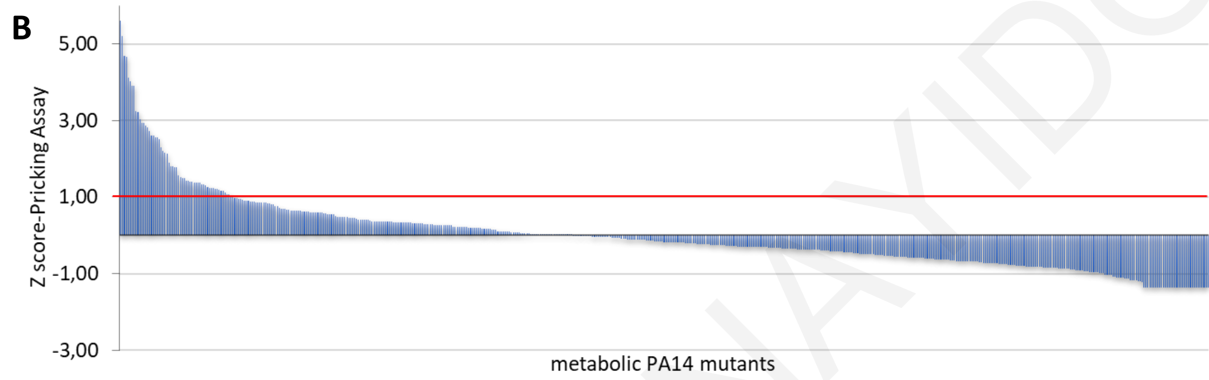

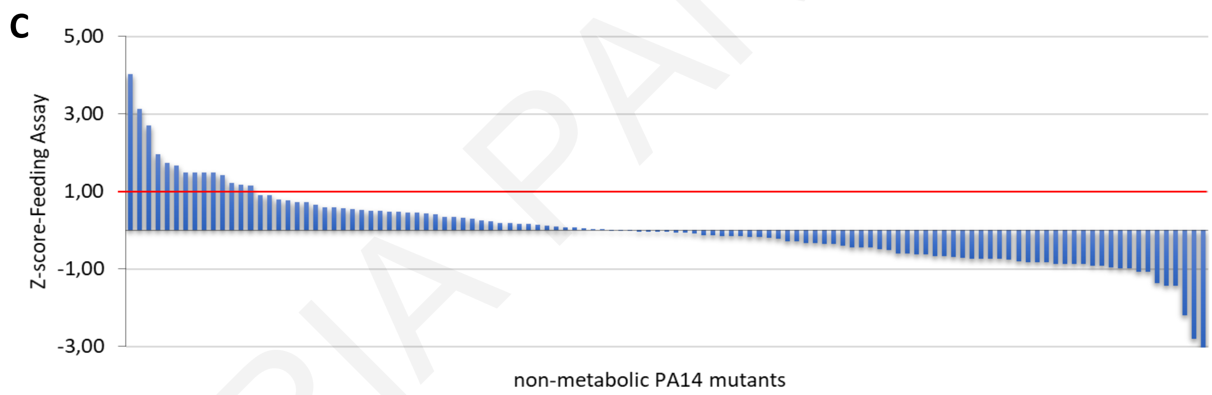

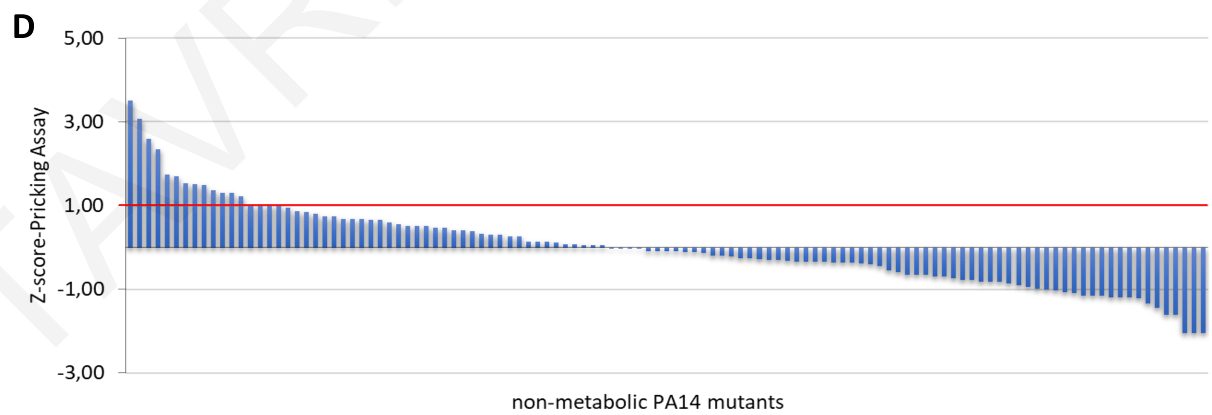

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Figure 6. Z-score analysis for the results of the feeding and pricking infection assays.
(A-B): Z-score analysis of the fly survival after infection of flies with 553 PA14 metabolic mutants (corresponding to 482 genes) using two independent assays: **(A)** Feeding Assay, **(B)** Pricking Assay. The time of 50% fly death (LT50%) was assessed for each mutant and condition and a Z-score analysis was used to select those with a score >1 for any of the two assays, from which upon retest, 78 were found significantly attenuated in virulence per Kaplan-Meier survival analysis with a log-rank test.
(C-D): Z-score analysis of the fly survival after infection of flies with 95 randomly selected non-metabolic PA14 mutants (corresponding to 94 genes) using two independent assays: **(C)** Feeding Assay, **(D)** Pricking Assay. The time of 50% fly death (LT50%) was assessed for each mutant and condition and a Z-score analysis was used to select those with a score >1 for any of the two assays, from which upon retest, 8 were found significantly attenuated in virulence per Kaplan-Meier survival analysis with a log-rank test.

4.2 Growth assessment of the virulence defective PA14 metabolic mutants

To determine which of the 78 attenuated PA14 metabolic mutants are virulence-related in flies and not growth-essential in culture or during infection, we extensively studied the 78 mutants for growth in two minimal media (one with glucose as a carbon source while the other additionally contained fly extract), as well as during infection at the respective infection model (would or oral) in which they were found to be attenuated in virulence.
Glucose supplemented minimal medium, offering the minimum nutrients required for colony growth, was initially used to identify 34 out of the 78 metabolic mutants that grow similarly to the wild-type strain and which, were categorized as prototrophs (**Figure 7 A-E & Table 2**). The remaining 44 metabolic mutants, did not grow at all or grow very slowly in this medium, being likely unable to use glucose to synthesize all the compounds needed for their growth. Nevertheless, if the needed compounds are available in flies during infection, the lack of virulence should be due to a direct effect of the mutants in virulence rather than in growth. To assess this possibility, we inoculated a glucose minimal medium supplemented with 5% fly extract to each of the 44 metabolic mutants. This medium offers all the nutrients the bacterium can find in flies in the absence of the host defense against the bacteria. We found 12 of the 44 PA14 metabolic mutants to grow similarly to the wild-type and considered them as conditional prototrophs (**Figure 8 A-E & Table 3 - Groups A, B**). The 8 virulence-related non-metabolic strains (**Table 1**) were able to grow in the latter assay and thus, we did not test their growth further (**Figure 9**).

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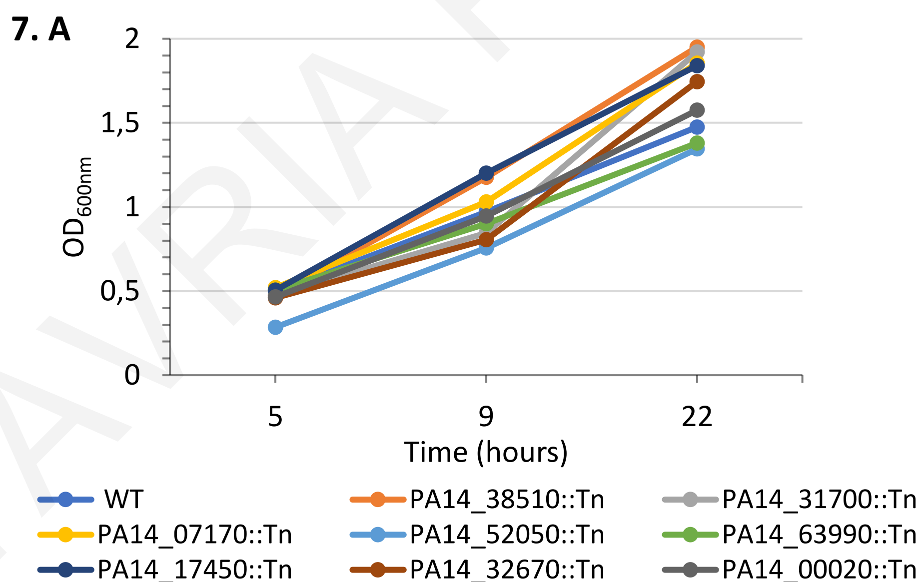
Moreover, we sought to examine the growth of the 78 selected metabolic mutants during initial colonization, to identify auxotrophic strains able to colonize the flies, but also to study the colonization ability of the prototrophs. We modified our wound and intestinal infection assays so that colonization efficiency can be reliably assessed. For the intestinal colonization assay, we infected flies for only one day with all flies being subsequently transferred to tubes with clean food, every day for 3 days. For the wound colonization assay, we injected the bacteria in the flies instead of pricking them with a tungsten needle to bypass the fly immune system that can easily eliminate the attenuated in virulence bacteria at the wound site. Calculating the number of retrievable bacteria per fly **(Figures 10 & 11)** we found that, 29 of the 34 prototrophs (**Table 2**) and 17 over the remaining 44 strains (**Table 3 - Group A, C**) were able to colonize the flies like the wild-type strain. In summary, we found 34 prototrophs (**Table 2**), 23 conditional prototrophs able to grow either in the host or in cultures supplemented with fly extract (**Table 3, Group A-C**) and 21 auxotrophs unable to grow in the host or in culture (**Table 3, Group D**). Accordingly, 11.8% (57/482) of the metabolic genes and 8.5% (8/94) of the non-metabolic were categorized as virulence-related, suggesting that many and functionally disparate metabolic genes are connected to *P. aeruginosa* virulence.



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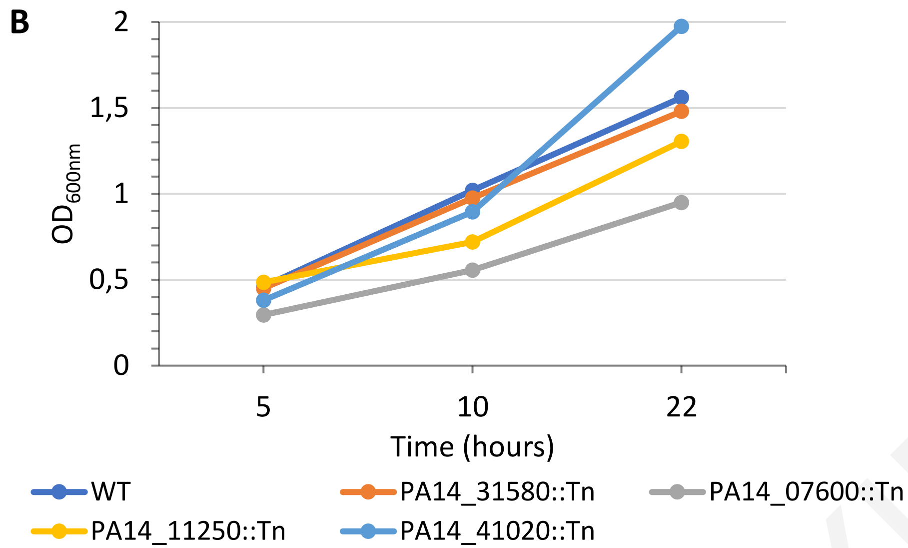

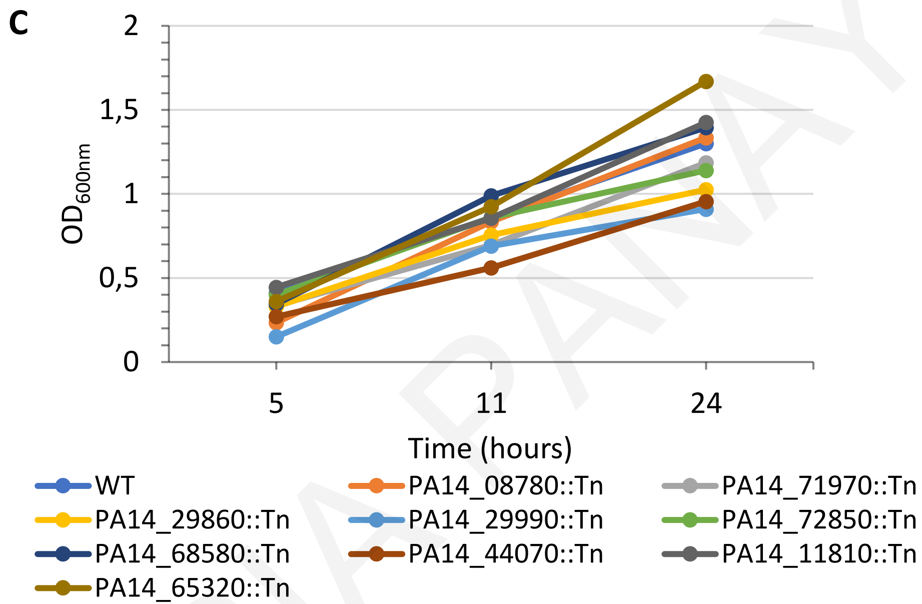

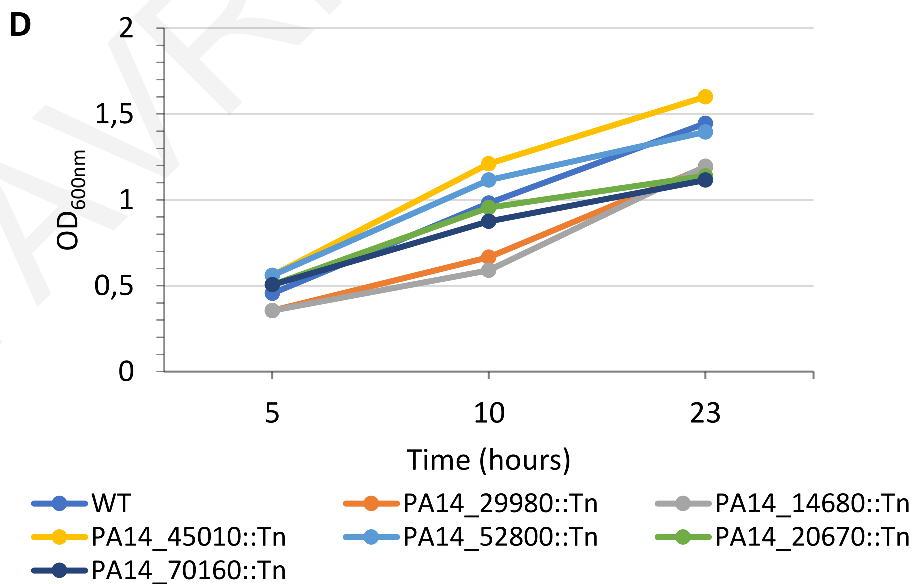

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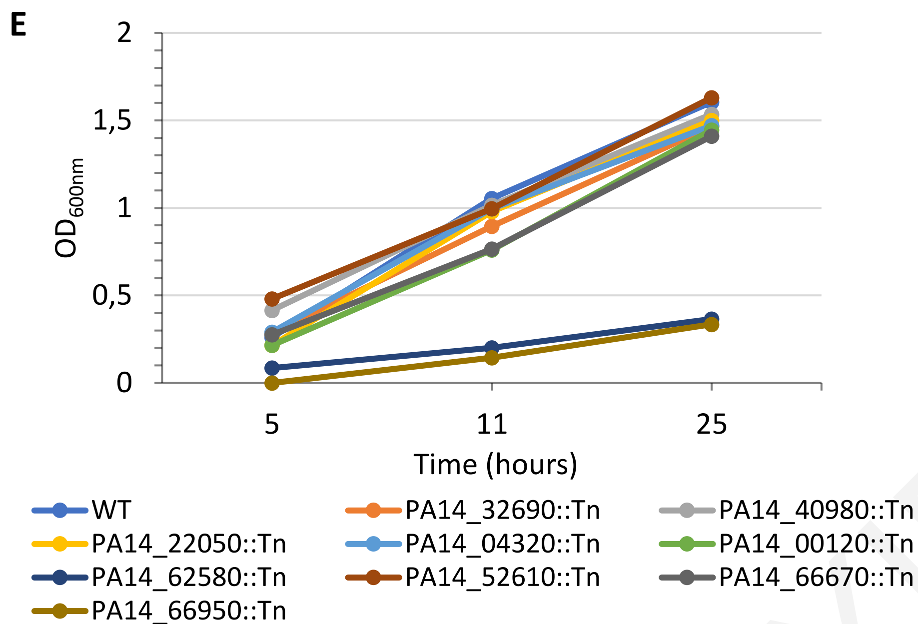
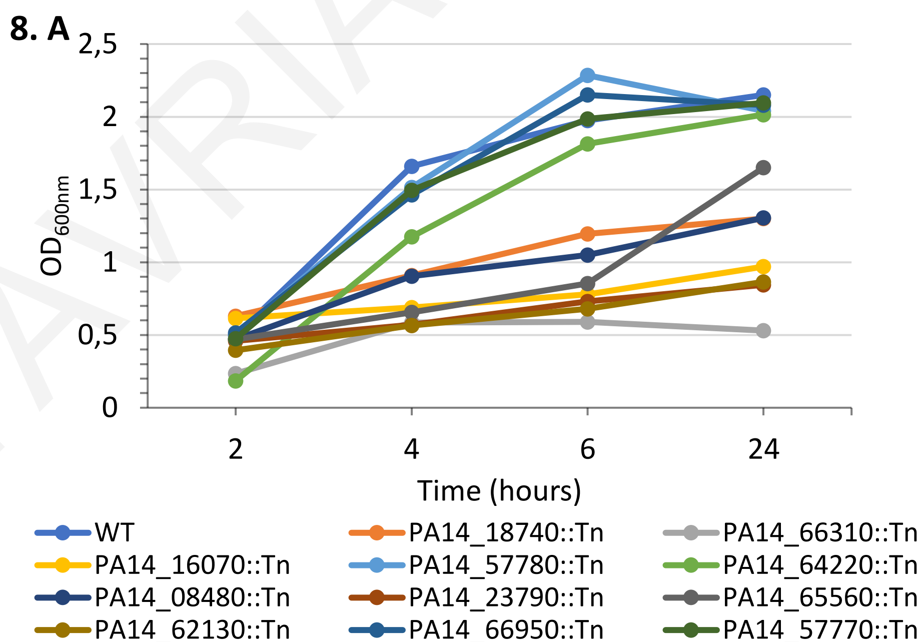


Figure 7. Growth of PA14 metabolic mutants in glucose minimal media (M9). (A-E) The growth of the selected metabolic mutants was assessed compared to the growth of the wild-type PA14, by measuring the optical density OD_{600nm} at three time points. The graphs are shown only the mutants that were able to grow in the glucose minimal medium similar or more slowly than the wild-type strain. In total 34 metabolic mutants were able to grow efficient in this medium and considered as prototrophs (see also **Table 2**). As shown in the last graph, the mutants PA14_62580 and PA14_66950 exhibited slow growth thus, they are not included in the list of prototrophs.



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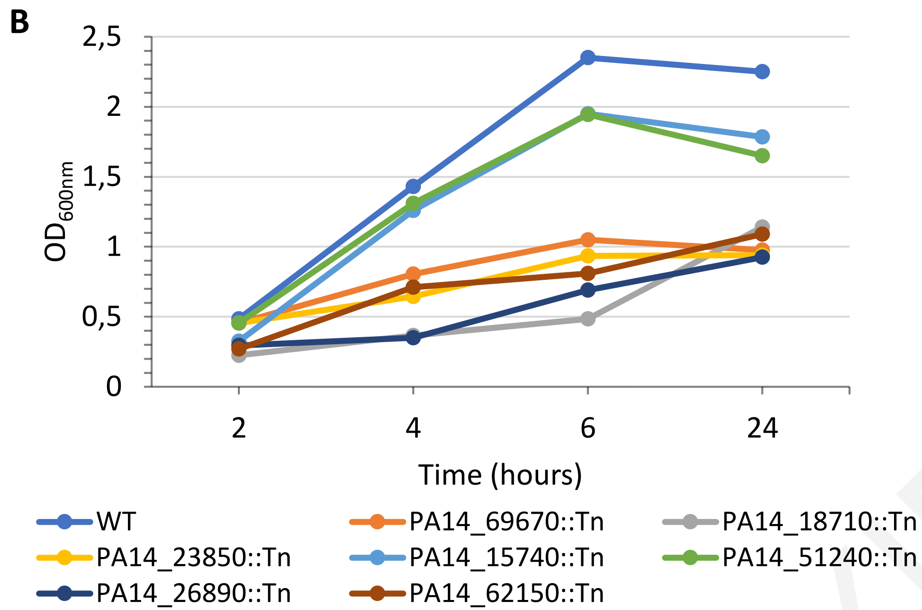

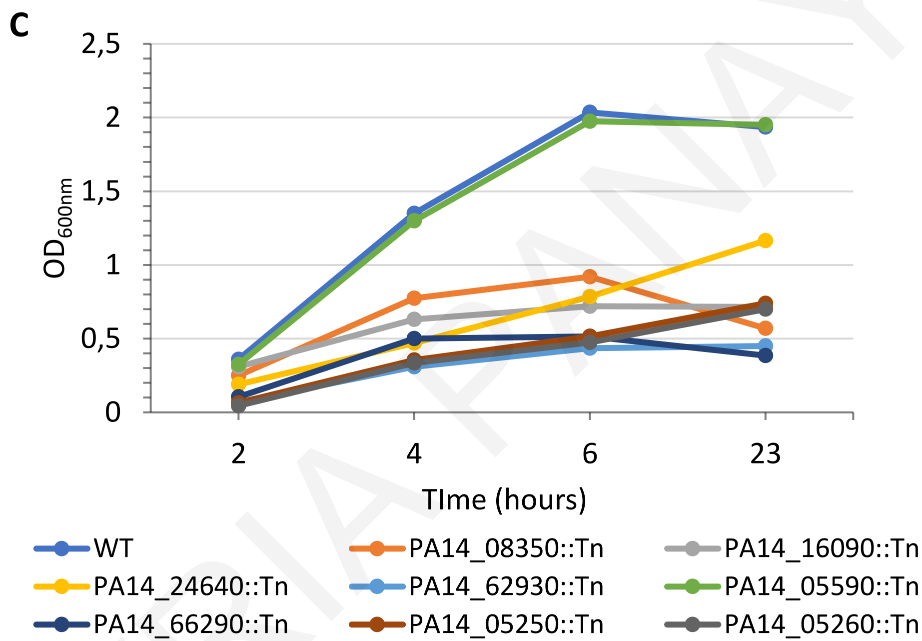

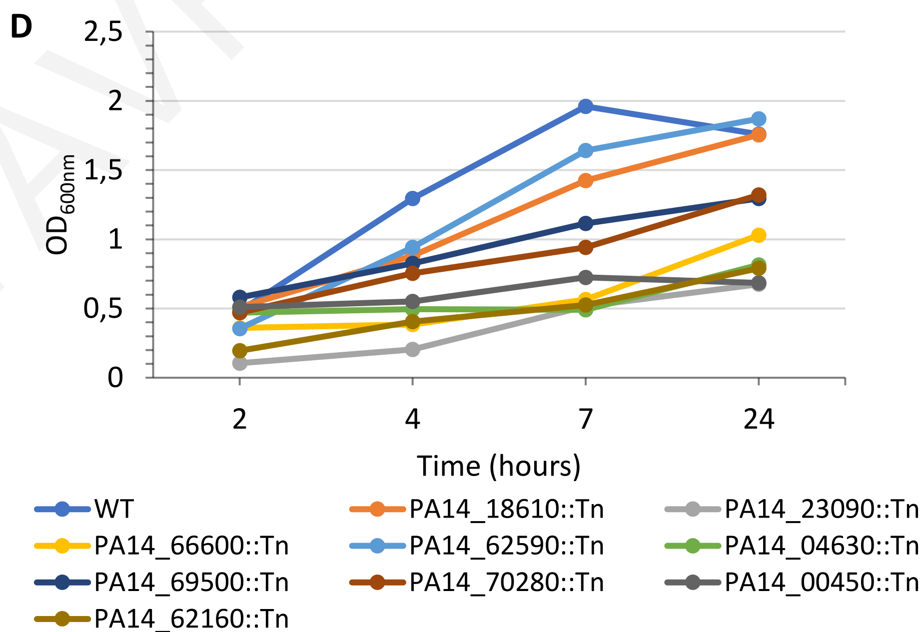

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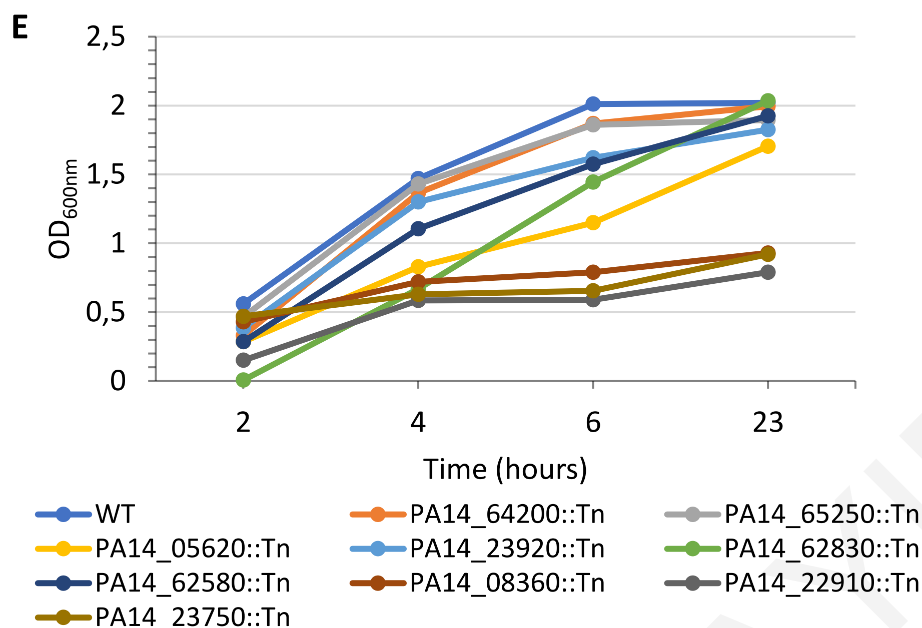

Figure 8. Growth of PA14 metabolic mutants in glucose minimal media supplemented with 5% fly extract. (A-E) The metabolic mutants that grow slowly or did not grow at all in glucose minimal medium, were also assessed in glucose minimal medium that additionally contained 5% fly extract. The optical density values for each metabolic mutant and for the wild-type PA14 were taken at four time points. Only 12 metabolic mutants that grown in cultures that had an optical density equal or higher of the value 1,5 at the time point of the 6 hours, were considered to grow efficiently in this medium (see also **Table 3**).

Table 1. Non-metabolic PA14 transposon mutants that found attenuated in flies during wound and/or oral infection. The table shows all the virulence-related non-metabolic mutants and the assay in which were found attenuated.

Non-metabolic PA14 Tn-mutants		
Attenuated in the Pricking Assay	Attenuated in the Feeding Assay	Attenuated in both assays
PA14_20730	PA14_10370	PA14_25110
PA14_27280	PA14_23990	
PA14_48830	PA14_70390	
PA14_58550		

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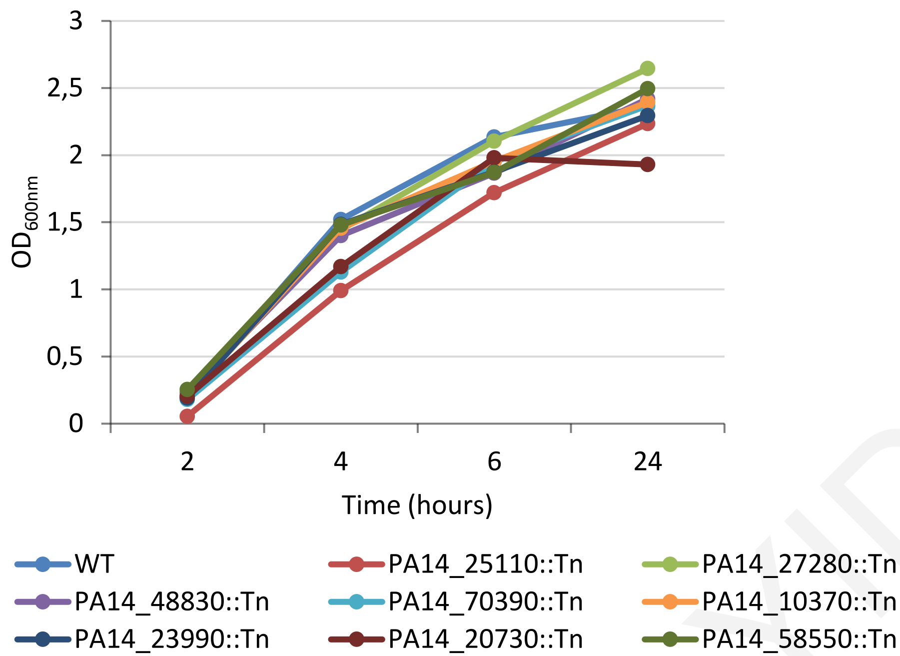
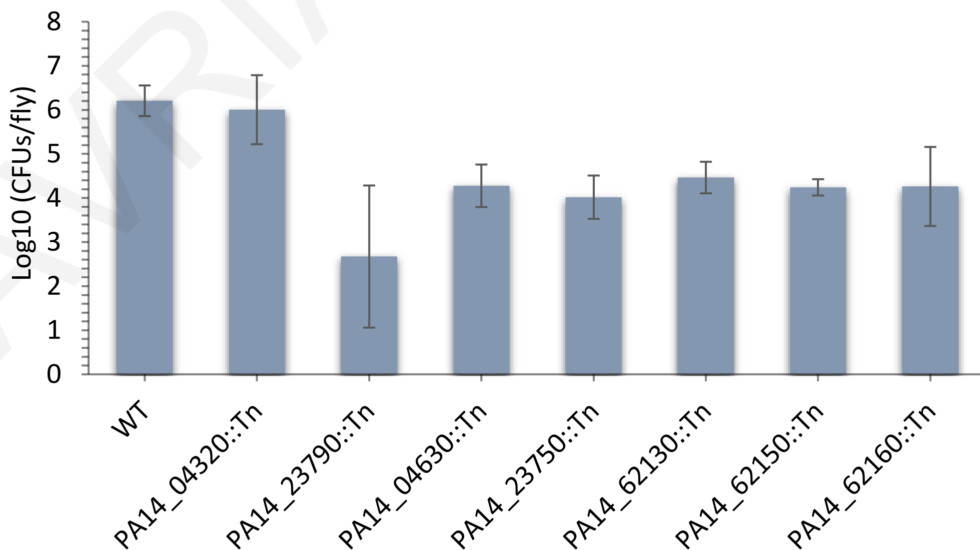


Figure 9. Growth of selected non-metabolic PA14 mutants in glucose minimal media supplemented with 5% fly extract. The growth of the selected non-metabolic mutants was assessed only in glucose minimal medium that additionally contained 5% fly extract to verify that these mutants have the potential to grow in flies. The optical density was measured at four time points. All of them were able to grow in this medium to the same extent as the wild-type PA14.



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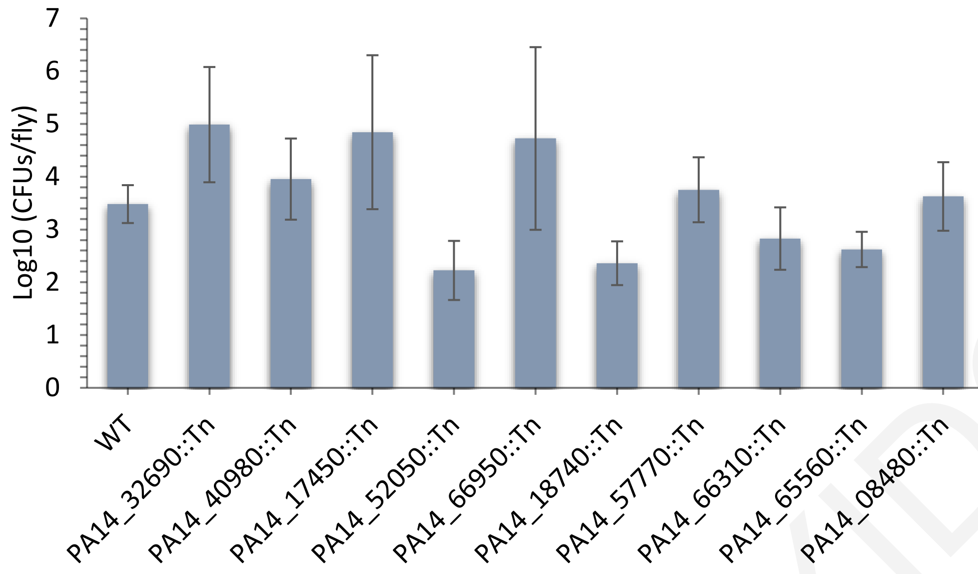

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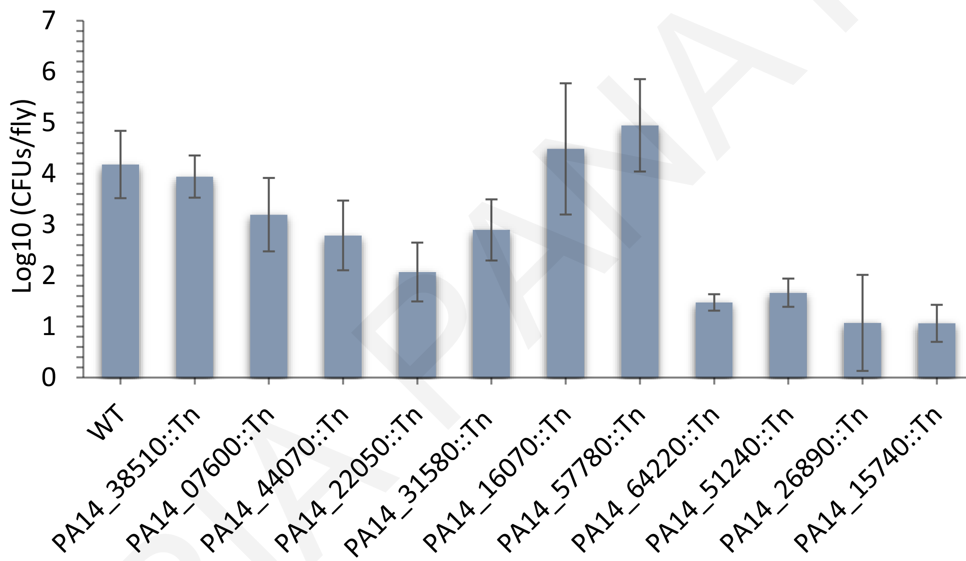

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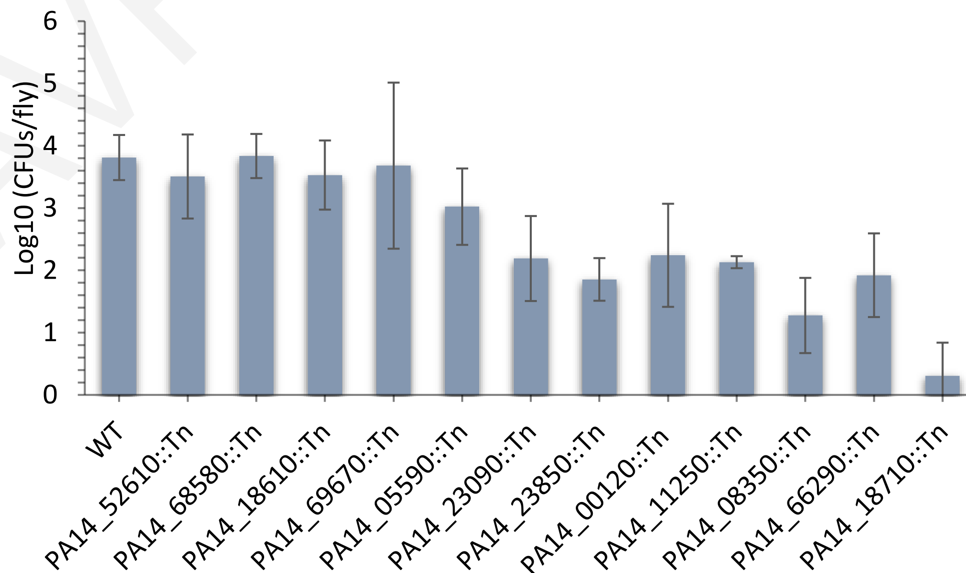

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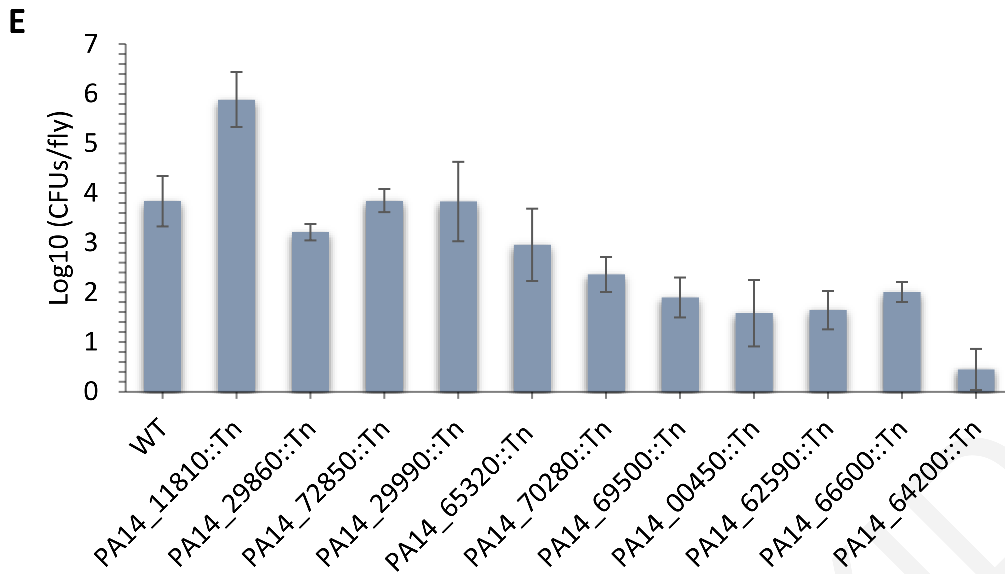

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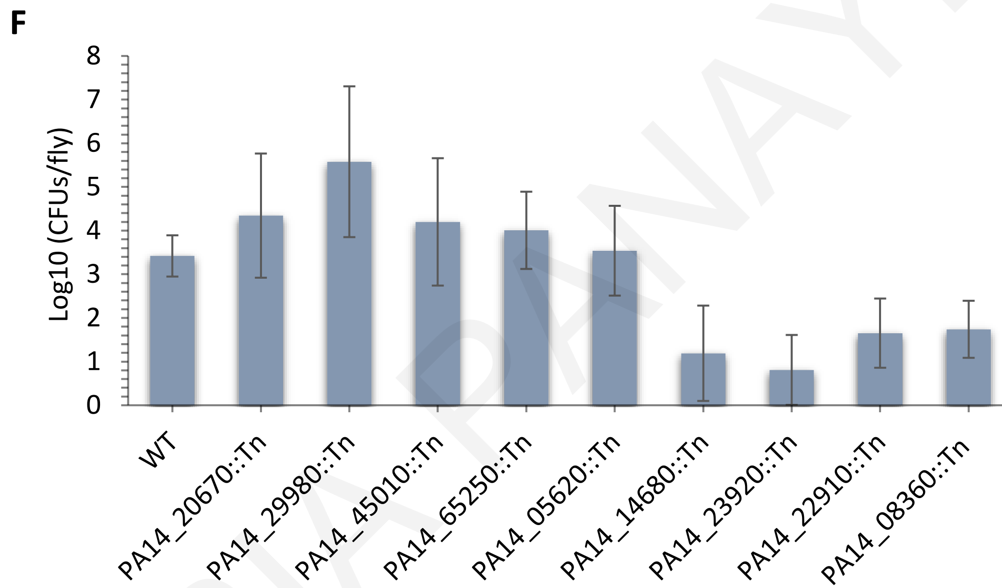

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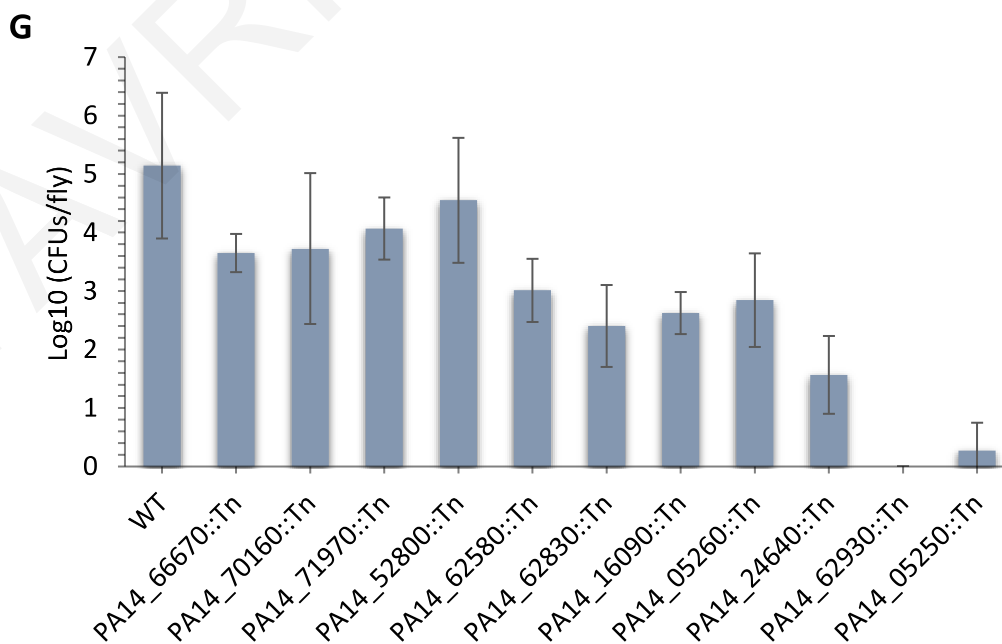

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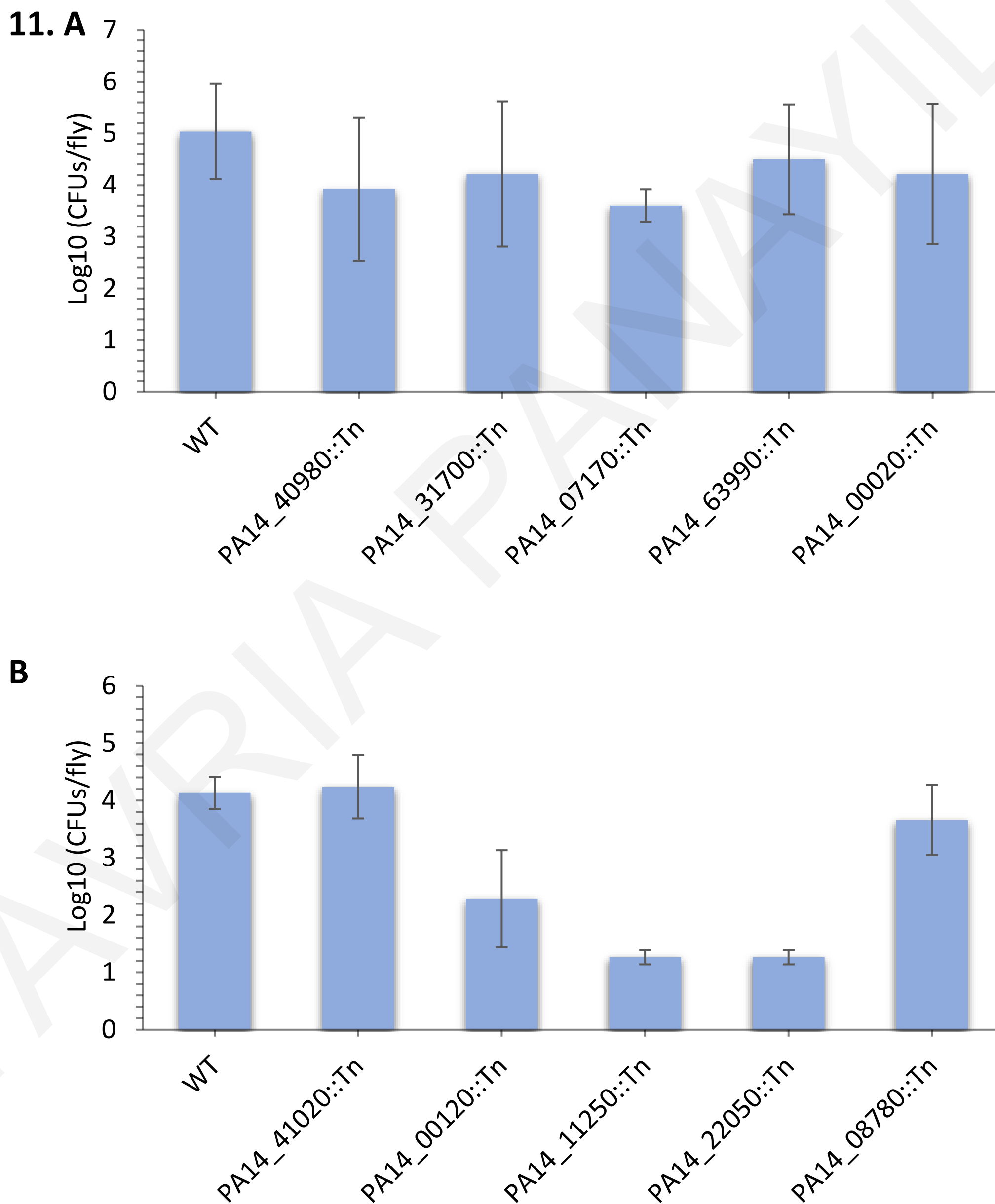
Figure 10. Intestinal colonization of the selected PA14 metabolic mutants.

(A-G) The colonization capacity, of the metabolic mutants that were initially selected by the feeding assay, as compared to the colonization of the wild-type strain, by counting CFUs per fly (average of 3 samples). Flies were previously orally infected with each strain, using a specially designed intestinal colonization assay. Only metabolic mutants with CFUs/fly values that do not differ statistically compared to those of the wild-type, were considered to colonize the flies efficiently (see **Tables 2 & 3**).



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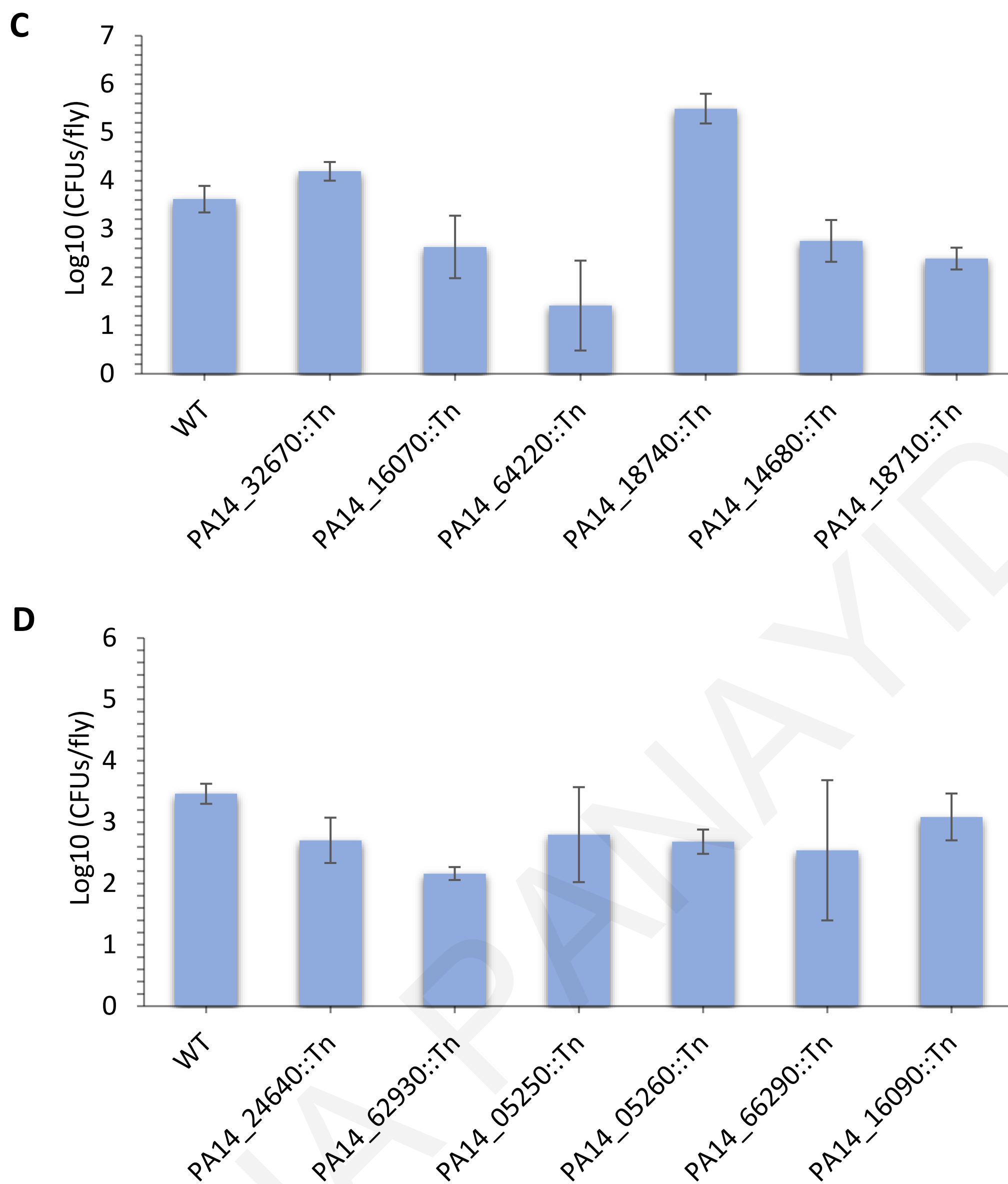

Figure 11. Wound colonization of the selected PA14 metabolic mutants.

(A-D) The colonization capacity, of the metabolic mutants that were initially selected by the pricking assay, as compared to the colonization of the wild-type strain, by counting CFUs per fly (average of 3 samples). Flies were previously injected with each strain. Only metabolic mutants with CFUs/fly values that do not differ statistically compared to those of the wild-type, were considered to colonize the flies efficiently (see **Tables 2 & 3**).

Table 2. Thirty-four virulence-related prototrophs based on growth in glucose minimal media. From the 78 virulence-defective PA14 metabolic mutants, 34 can grow like the wild-type in the M9 medium, indicating a connection of the corresponding genes with virulence.

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	Metabolic PA14 mutants	*In vitro* growth	*In vivo* growth (colonization)	
#		**Minimal medium (M9)**	**Intestinal**	**Wound**
1*	PA14_00120 (lipid A biosynthesis lauroyl acyltransferase)	+	-	-
2	PA14_04320 (ilvA1)	+	+	NT
3	PA14_07600 (folk)	+	+	NT
4*	PA14_11250 (hypothetical protein)	+	-	-
5	PA14_11810 (aldehyde dehydrogenase)	+	+	NT
6*	PA14_14680 (suhB)	+	-	-
7	PA14_17450 (surE)	+	+	NT
8*	PA14_22050 (htrB)	+	-	-
9	PA14_20670 (glutamine synthetase)	+	+	NT
10	PA14_29860 (nuoM)	+	+	NT
11	PA14_29980 (nuoE)	+	+	NT
12	PA14_29990 (nuoD)	+	+	NT
13	PA14_31580 (acyl-CoA dehydrogenase)	+	+	NT
14	PA14_32690 (gtdA)	+	+	NT
15	PA14_38510 (hmgA)	+	+	NT
16*	PA14_40980 (enoyl-CoA hydratase)	+	+	+
17	PA14_44070 (gltA)	+	+	NT
18	PA14_45010 (hyi)	+	+	NT
19	PA14_52050 (purN)	+	-	NT
20	PA14_52610 (hypothetical protein)	+	+	NT
21	PA14_52800 (acsA)	+	+	NT
22	PA14_65320 (miaA)	+	+	NT
23	PA14_66670 (ponA)	+	+	NT
24	PA14_68580 (pckA)	+	+	NT
25	PA14_70160 (bioA)	+	+	NT
26	PA14_71970 (wbpW)	+	+	NT
27	PA14_72850 (glutamine synthetase)	+	+	NT
28	PA14_00020 (dnaN)	+	NT	+
29	PA14_07170 (epd)	+	NT	+
30	PA14_08780 (rpoC)	+	NT	+
31	PA14_31700 (pgsA)	+	NT	+

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32	PA14_32670 (hypothetical protein)	+	NT	+
33	PA14_41020 (adi)	+	NT	+
34	PA14_63990 (speA)	+	NT	+
Feeding assay-selected: 1-27; Those selected in both assays are shown with asterisk (*); Pricking assay-selected only: 28-34; NT: Not Tested				

Table 3. Forty-four virulence-defective mutants: 23 conditional prototrophs & 21 auxotrophs. These metabolic mutants are unable to grow in glucose minimal medium (M9) but 23 of them can conditionally grow *in vitro* and/or *in vivo*, like the wild-type strain, if they find in the medium or in the host the missing compounds needed for their growth. The rest 21 do not grow like the wild-type in any condition, probably because the missing compounds needed for their growth, are not available in the conditions we used.

#	Major Pathways	Metabolic PA14 mutants	*In vitro* growth	*In vivo* growth (colonization)		
			Glucose minimal medium (M9) with 5% Fly Extract	Intestinal	Wound	
1	Histidine metabolism	PA14_57770 (hisC1)	+	+	NT	**Group (A)** **(# 6 mutants)** Efficient growth in **glucose minimal medium with 5% fly extract** and ***in vivo***
2		PA14_57780 (hisD)	+	+	NT	
3		PA14_66950 (hisE)	+	+	NT	
4		PA14_65250 (hisX)	+	+	NT	
5	One carbon pool by folate	PA14_05590 (metF)	+	+	NT	
6	Pantothenate and CoA biosynthesis	PA14_62580 (panB)	+	+	NT	
7		PA14_62590 (panC)	+	-	NT	**Group (B)** **(# 6 mutants)** Efficient growth only in **glucose minimal medium with 5% fly extract**
8	Purine metabolism	PA14_51240 (purC)	+	-	NT	
9*		PA14_64220 (purD)	+	-	-	
10		PA14_23920 (purF)	+	-	NT	
11*		PA14_64200 (purH)	+	-	NT	
12		PA14_15740 (purL)	+	-	NT	
13*	Pyrimidine metabolism	PA14_05260 (pyrB)	-	+	-	**Group (C)** **(# 11 mutants)** Efficient growth only ***in vivo***
14*		PA14_05250 (pyrC)	-	-	+	
15*	Glycine, serine and threonine metabolism	PA14_16070 (hom)	-	+	+	
16*		PA14_16090 (thrC)	-	-	+	
17	Arginine biosynthesis	PA14_08480 (argC)	-	+	NT	
18*		PA14_18740 (argG)	-	+	NT	
19		PA14_18610 (argF)	-	NT	+	
20	Glycolysis / Gluconeogenesis	PA14_66310 (aceF)	-	+	NT	
21*		PA14_66290 (aceE)	-	+	NT	
22	Cysteine and methionine metabolism	PA14_05620 (sahH)	-	-	+	
23	Lysine biosynthesis	PA14_69670 (lysA)	-	+	NT	
24		PA14_00450 (trpB)				

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25	Phenylalanine, tyrosine and tryptophan biosynthesis	PA14_08360 (trpC)	**Group (D)** **(# 21 mutants)** **Slow growth** compared to the wild-type PA14 in all the above assays
26		PA14_08350 (trpD)	
27		PA14_23850 (trpF)	
28		PA14_66600 (aroB)	
29	Valine, leucine and isoleucine biosynthesis	PA14_62130 (ilvC)	
30		PA14_04630 (ilvD)	
31		PA14_62150 (ilvH)	
32		PA14_62160 (ilvI)	
33		PA14_23790 (leuB)	
34		PA14_23750 (leuC)	
35*	Pyrimidine metabolism	PA14_18710 (pyrC)	
36*		PA14_24640 (pyrD)	
37		PA14_26890 (pyrF)	
38*		PA14_62930 (carA)	
39	Pentose phosphate pathway	PA14_22910 (edd)	
40		PA14_23090 (edaA)	
41	Arginine biosynthesis	PA14_70280 (argB)	
42		PA14_69500 (argH)	
43	Glycine, serine and threonine metabolism	PA14_65560 (serB)	
44	Glycolysis / Gluconeogenesis	PA14_62830 (tpiA)	
*: Found attenuated in both assays (pricking and feeding) | NT: Not Tested			

4.3 Virulence-related metabolic genes of *P. aeruginosa* PA14 belong in central metabolic pathways and many of them are necessary for full virulence in mice

According to the KEGG database, the selected 57 virulence-related metabolic genes, belong to 7 central metabolic pathways (**Figure 12**). Assessing single mutant representatives, from each metabolic pathway as defined in the KEGG database, in an acute murine lung infection model, we found 15 mutants to be defective in virulence (**Figure 13**). The mutants belong primarily in amino acid metabolism [PA14_57770 (*hisC1*), PA14_57780 (*hisD*), PA14_65250 (*hisX*), PA14_32690 (*gtdA*), PA14_16070 (*hom*)], nucleotide metabolism [PA14_17450 (*surE*), PA14_64220 (*purD*), PA14_52050 (*purN*)] and metabolism of co-factors and vitamins [PA14_05590 (*metF*), PA14_07170 (*epd*), PA14_07600 (*folk*)] (**Table 4**).

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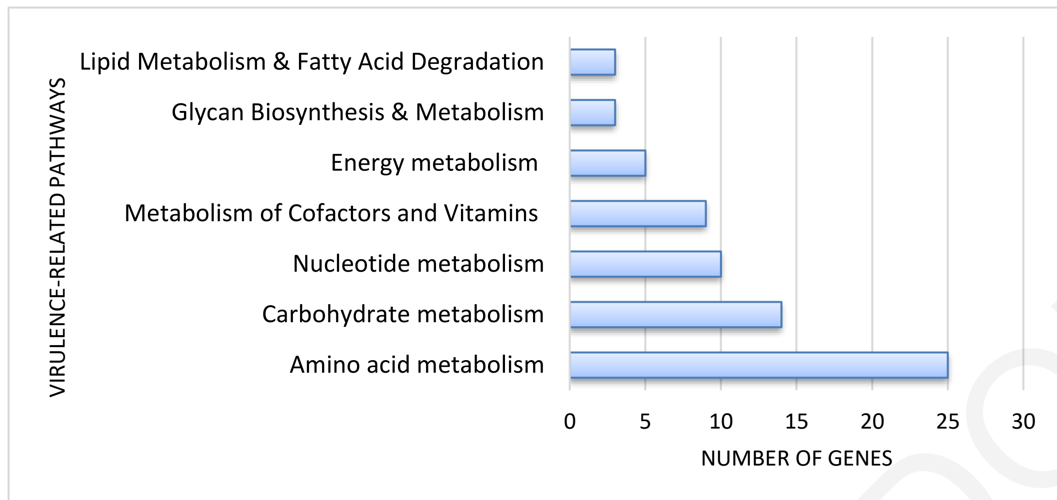

Figure 12. Central metabolic pathways of the 57 PA14 virulence-related metabolic genes. The 7 central metabolic pathways of the selected 57 virulence-related metabolic genes, according to the KEGG database. The total number of genes in the graph is greater than 57 because most of the metabolic genes are implicated in more than one metabolic pathway which in turn belongs in different categories of central metabolic pathways.

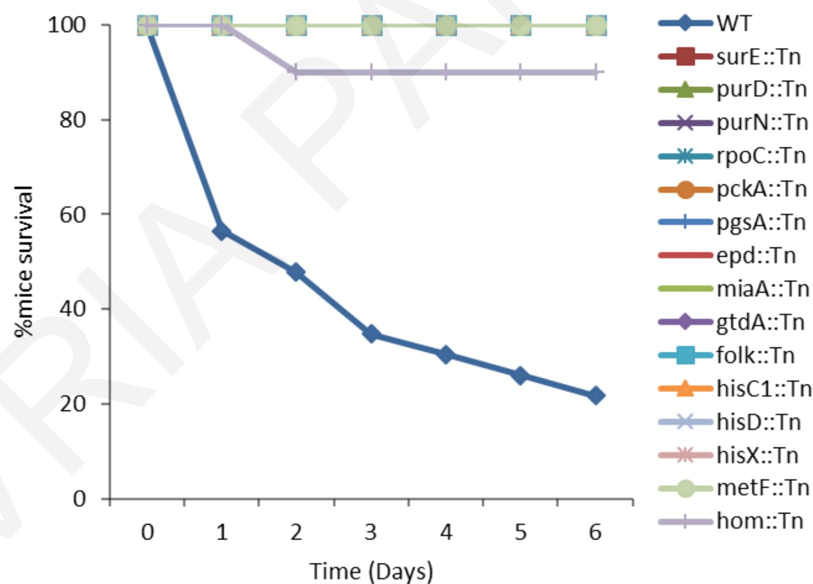

Figure 13. Survival kinetics of mice after intranasal inoculation with PA14 metabolic mutants. The graph shows the % mice survival after intranasal inoculation with PA14 metabolic mutants and the wild-type strain. Twenty-microliter aliquots of a bacterial solution containing $2x10^7$ bacteria were administered intranasally to each mouse. Infected mice were monitored for 6 days (n=10-11).

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Table 4. Pathways of the 15 PA14 metabolic genes corresponding to mutants that found attenuated in mice lung infection. The names of the metabolic genes are listed in the first column. The second column shows the metabolic pathways in which each gene is involved based on the KEGG database, while the last column shows the major central metabolic pathways in which those pathways belong. The transposon insertion sites of the 15 virulence-related PA14 metabolic genes are shown in **Figure 29 (A-N)**.

#	Genes/Names	Major Metabolic Pathways (based on KEGG database)	Major Central Metabolic Pathways
1	PA14_17450 (*surE*)	Purine, Pyrimidine & Nicotinate and nicotinamide metabolism	Nucleotide metabolism Metabolism of cofactors and vitamins
2	PA14_64220 (*purD*)	Purine metabolism	
3	PA14_52050 (*purN*)	Purine metabolism, One carbon pool by folate	
4	PA14_08780 (*rpoC*)	RNA polymerase Protein families: genetic information processing (Transcription machinery)	
5	PA14_57770 (*hisC1*)	Histidine, Tyrosine & Phenylalanine metabolism Phenylalanine, tyrosine and tryptophan biosynthesis etc.	Amino acid metabolism
6	PA14_57780 (*hisD*)	Histidine metabolism	
7	PA14_65250 (*hisX*)	Histidine metabolism	
8	PA14_32690 (*gtdA*)	Tyrosine metabolism	
9	PA14_16070 (*hom*)	Glycine, serine and threonine metabolism Cysteine and methionine metabolism Lysine biosynthesis	
10	PA14_05590 (*metF*)	One carbon pool by folate	Metabolism of cofactors and vitamins
11	PA14_07170 (*epd*)	Vitamin B6 metabolism	
12	PA14_07600 (*folk*)	Folate Biosynthesis	
13	PA14_68580 (*pckA*)	Glycolysis / Gluconeogenesis, Citrate cycle (TCA cycle), Pyruvate metabolism	Carbohydrate metabolism
14	PA14_65320 (*miaA*)	tRNA dimethylallyltransferase Protein families: genetic information processing	Transfer RNA biogenesis
15	PA14_31700 (*pgsA*)	Glycerophospholipid metabolism	Lipid metabolism

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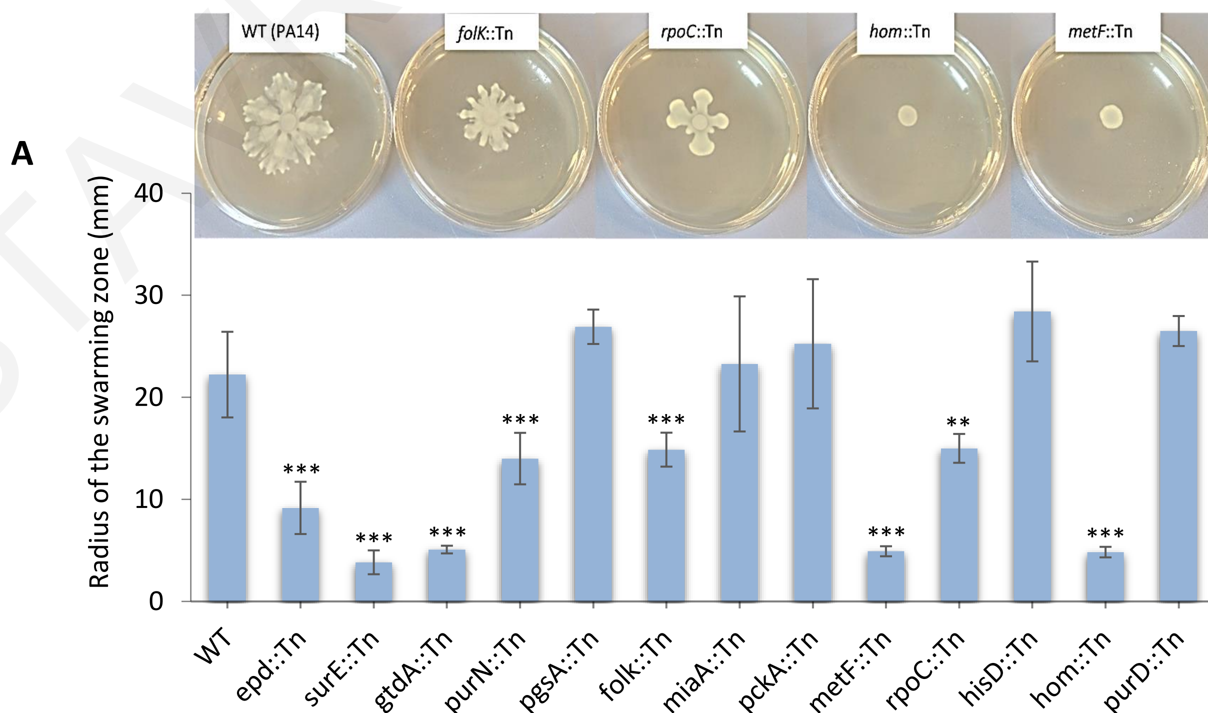
4.4 Virulence-related metabolic genes of *P. aeruginosa* PA14 are compromised in various aspects of virulence

To identify the connection between *P. aeruginosa* metabolism and virulence, we examined the virulence factor production in 13 of the 15 virulence-related metabolic mutants that were validated in the mouse infection assay. This was because 3 of the 15 selected mutants were histidine mutants (*hisC1*, *hisD*, and *hisX*) thus we examined the virulence factor production only in one of them (*hisD* mutant), assuming functional redundancy among them. *HisD* is located downstream of the other two histidine genes in the histidine biosynthesis pathway and consequently, we assumed that any phenotypes of the *hisD* mutant would be representative of those of the other two histidine mutants. Additionally, both *hisC1* and *hisX* genes have orthologous genes, thus any phenotypes of the corresponding mutants could be recovered by their orthologs.

We studied the expression of virulence factors related to the acute infection including, bacterial motility and T3SS, as well as the expression of quorum-sensing genes.

4.4.1 Role of PA14 metabolic genes in motility

Bacterial motility is very important for *P. aeruginosa* virulence [43]. We examined if the disruption of *P. aeruginosa* metabolic genes affecting its motility. We tested two types of motility: swarming and twitching for the 13 PA14 metabolic Tn-mutants found attenuated in both flies and mice. The mutants *epd*, *surE*, *gtdA*, *purN*, *folK*, *metF*, *hom* and *rpoC*, were noticeably defective for swarming motility compared to the wild-type strain ($P < 0.001$ for the first 7 and $P < 0.01$ for the last one), while *pgsA* and *purD*, exhibited increased swarming motility compared to the wild-type ($P < 0.05$) (**Figure 14 A**).



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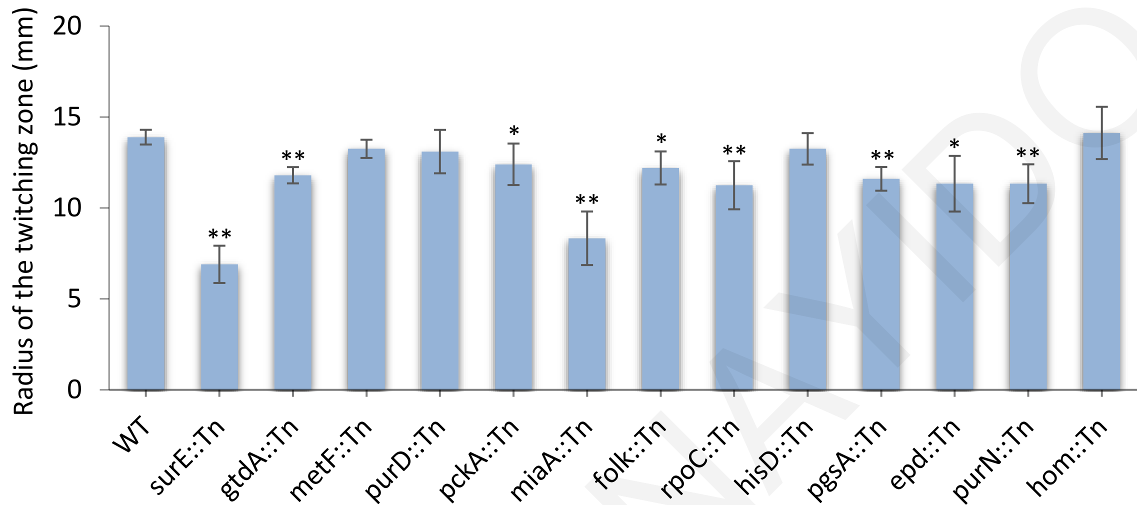
Regarding the twitching motility, the mutants *surE*, *gtdA*, *miaA*, *rpoC, pgsA* and *purN* exhibited a strong decrease in their ability to twitch compared to the wild-type ($P < 0.01$) (**Figure 14 B**), but also the *pckA*, *folk*, and *epd* mutants were significantly defective twitching motility ($P < 0.05$) (**Figure 14 B**). Only *hisD* and *purD* Tn-mutants were not affected in any of the two motilities (**Figure 14 B**).

B



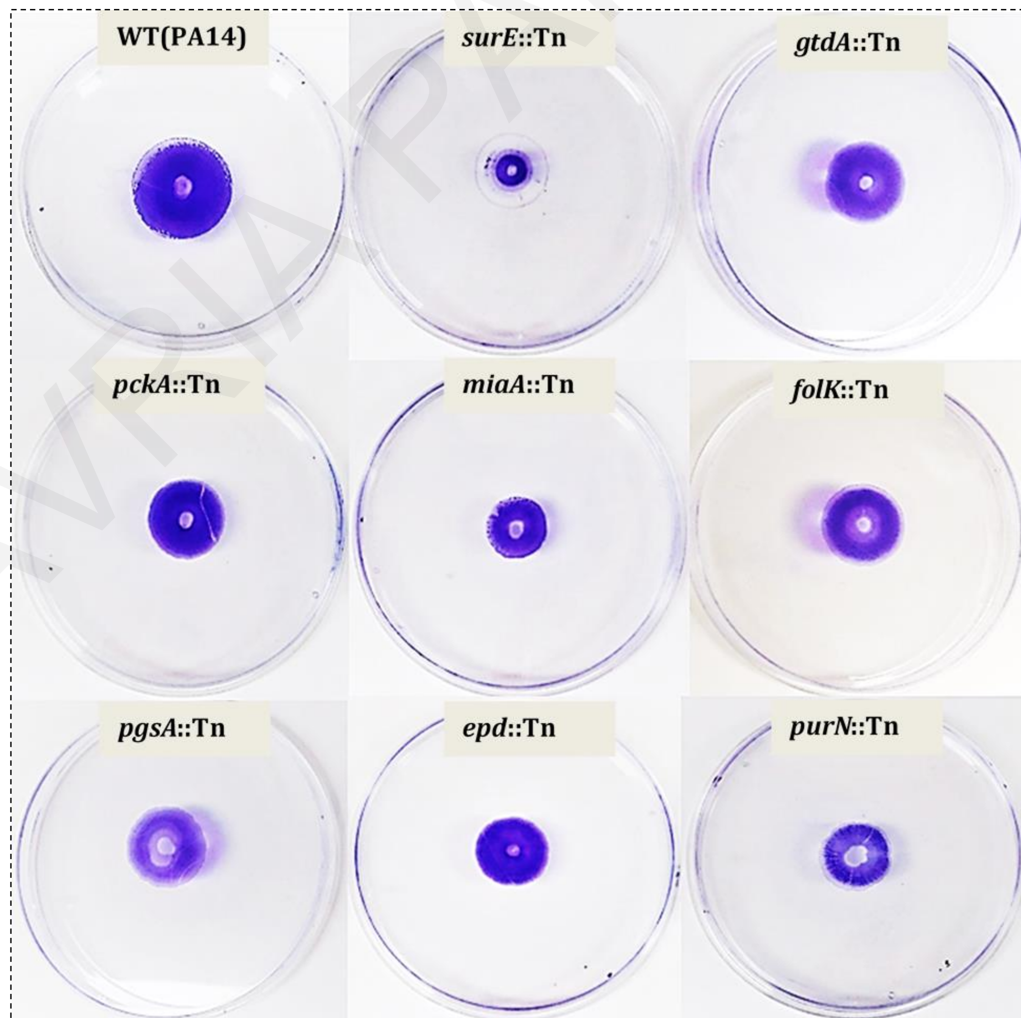


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Figure 14. Swarming and twitching phenotypes of PA14 metabolic mutants.

(**A)** Swarming motility was measured by the length of the swarming zone from the center of the plate after the inoculation of the cells at the center of the agar media and incubation at 37°C for 24 h. The results are from 3 independent experiments. n=3-13; **, P < 0.01; ***, P < 0.001 compared to WT by Mann-Whitney *U* test. Representative photos of swarm-negative phenotypes or defective swarming motility, of PA14 metabolic mutants compared to the wild-type, are shown above the graph. **(B)** For the twitching motility, the cells were stab-inoculated onto LB twitching plates (1% agar). The plates were incubated at 37 °C for 48 hours. The agar was removed, and the twitching zone was revealed by staining with crystal violet. Twitching motility was measured by the length of the twitching zone from the center of the plate. The results are from 3 independent experiments. n=4-7; *, P < 0.05; **, P < 0.01 compared to WT by Mann-Whitney *U* test. Representative photos of defective twitching motility, of PA14 metabolic mutants compared to the wild-type, are shown below the graph.

4.4.2 Role of PA14 metabolic genes in the expression of T3SS and other virulence genes

Gram-negative bacterial pathogens have evolved multiple protein secretion systems that facilitate the infection of eukaryotic hosts [116]. *P. aeruginosa* utilizes its type III secretion system (T3SS) to enhance its pathogenicity by injecting cytotoxic effector proteins into the host cells [28]. T3SS is regulated transcriptionally and post-transcriptionally in response to host cell contact and environmental Ca^{2+} levels.

We examined the expression of the T3SS regulatory gene *exsC* and the effector *exoT*, in the 13 Tn-mutants, under Ca^{2+} limiting conditions (5mM EGTA). We observed reduced expression of the *exoT* in 10 of the 13 selected Tn-mutants and overexpression in 1 of them, compared to the wild-type (**Figure 15 A**). Similarly, the expression of *exsC* is reduced in 5 of these mutants, with only *purD* and *gtdA* remaining unaffected in the expression of any of the two genes (**Figure 15 B**). *PurD* and *gtdA*, as well as the *miaA*, *rpoC*, *surE* and *purN* mutants, were nevertheless compromised in the expression of the type IV pilus biogenesis gene *pilA* (**Figure 15 D**), while *pa1L* gene, which is controlled by the quorum-sensing is significantly affected by *miaA*, *rpoC* and tentatively *purD* (**Figure 15 C**). *miaA*, is a tRNA isopentenyl transferase, a tRNA modification enzyme important for translation efficiency, while *rpoC* is the DNA-directed RNA polymerase beta subunit that has an important role in transcription. Our results suggest that the disruption in *miaA* and *rpoC* genes as well as in other metabolic genes, impact acute virulence by compromising various aspects of virulence.

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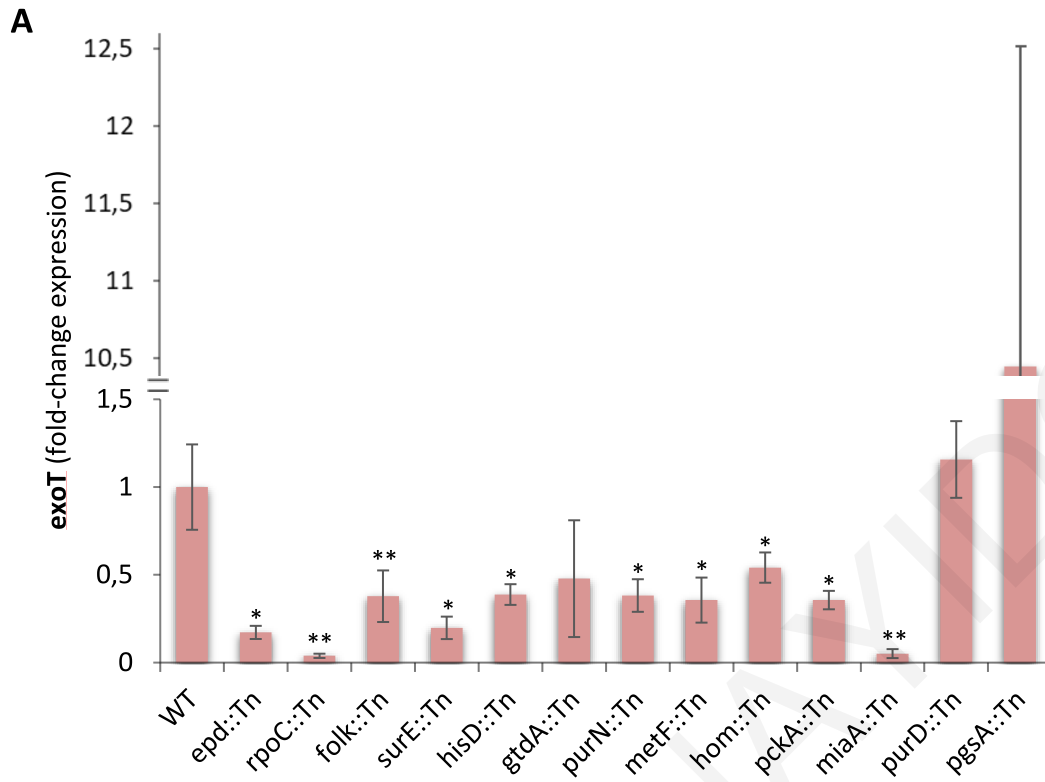

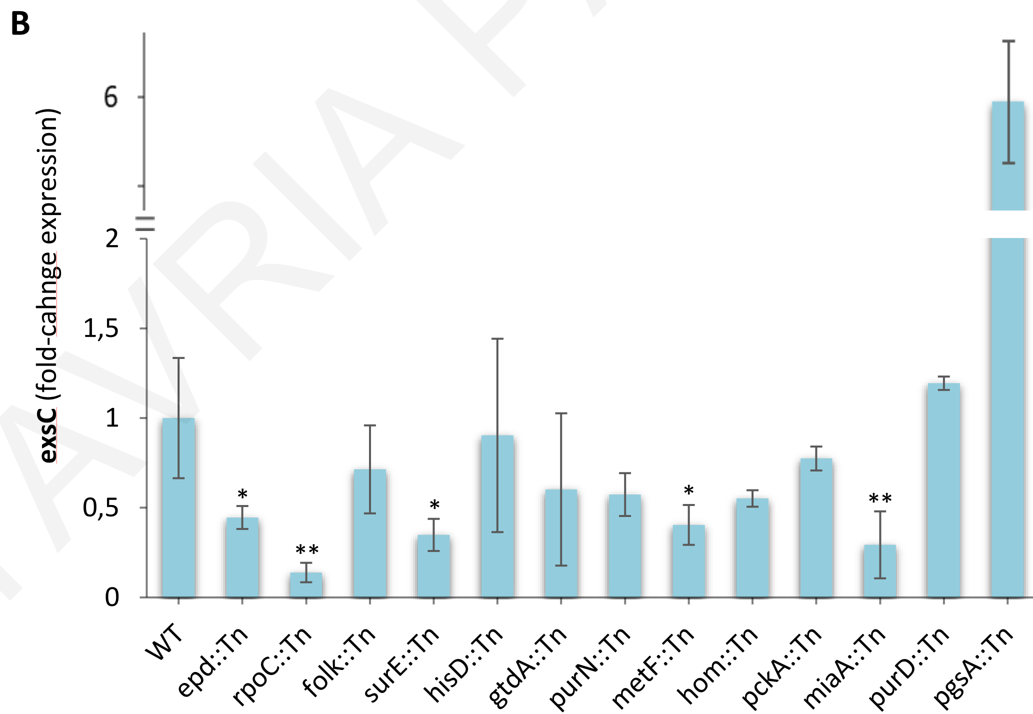

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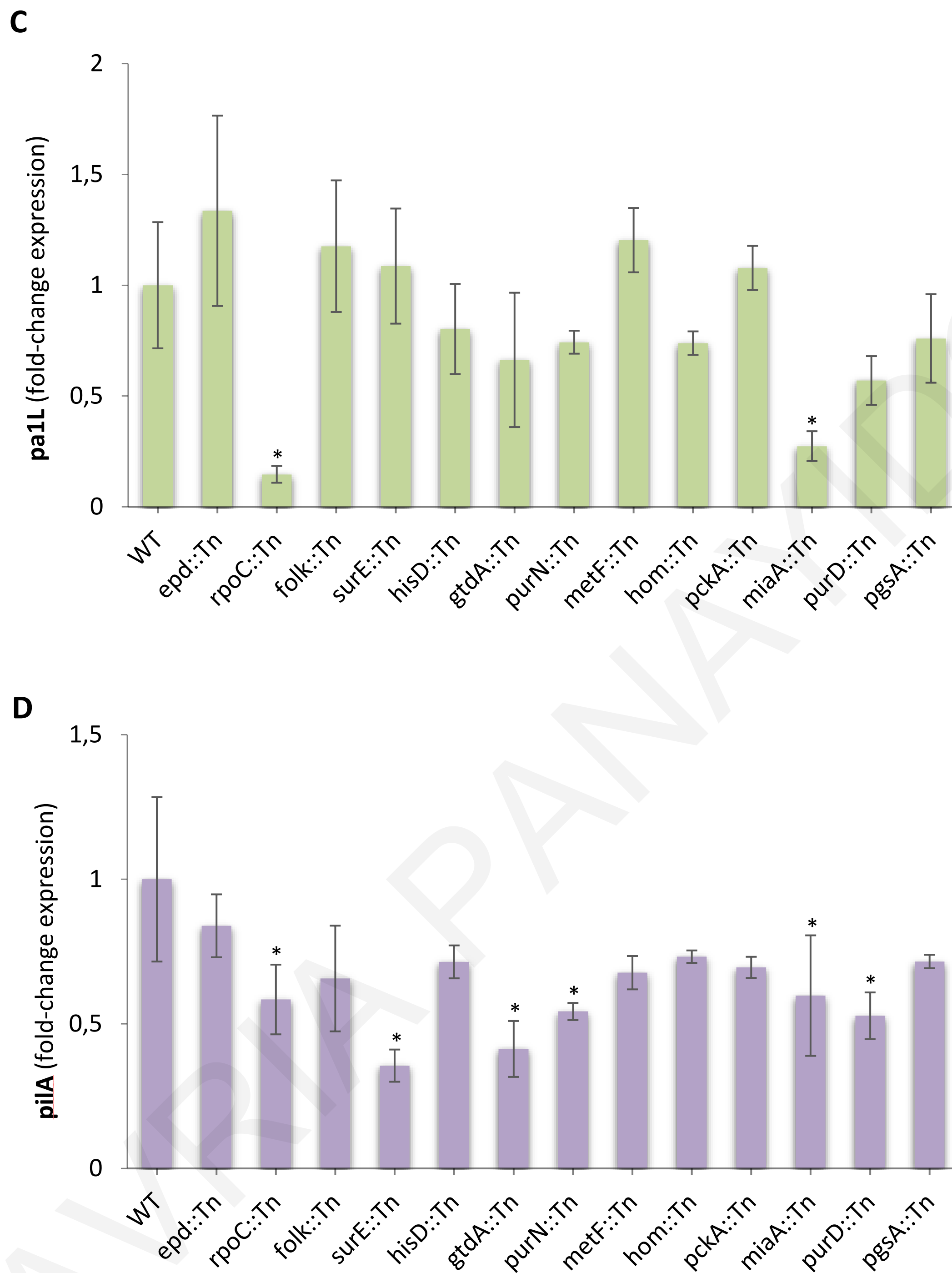

Figure 15. PA14 metabolic genes are required for the expression of various virulence factors. (A, B) Expression of T3SS genes in PA14 metabolic mutants. **(C, D)** Expression of *pa1L* and type IV pilus gene *pilA* in PA14 metabolic mutants. Bacteria were grown to an OD_{600nm} of 1 in LB with 5 mM EGTA before RNA extraction and cDNA synthesis.

[*, P < 0.05; **, P < 0.01 compared to WT by Mann-Whitney *U* test (n=3-7)].

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4.5 The putative stationary-phase survival protein *surE*

Among the 13 selected virulence-related PA14 metabolic genes, we pinpoint a novel gene, encoding *P. aeruginosa* putative stationary-phase survival protein-*surE*, the homolog of *Escherichia coli surE*, a nucleotidase with a broad-spectrum phosphatase activity against nucleotide monophosphates (e.g. GMP, AMP, IMP, XMP, UMP, CMP) [117]. This gene, while dispensable for growth, is essential for full virulence in a *Drosophila* oral (feeding assay) and an acute murine lung infection assay. We hypothesize that pinpointing genes dispensable for bacterial growth *in vitro*, serves two purposes: (a) indicate aspects of bacterial metabolism required for virulence but not for housekeeping functions, and (b) targeting such genes pharmacologically is less likely to affect the function of healthy microbiota bearing homologs of these genes.

The structural and functional characteristics of the *surE* protein have been described in other bacterial species [118-120], however, its virulence was not extensively studied. For this reason, we focused on the *surE* gene for further investigation.

4.6 *surE* regulates swimming, swarming and twitching motility

As described in the introduction, in addition to other virulence factors, motility also plays a central role in the pathogenesis of *P. aeruginosa*. Specifically, twitching motility enables this bacterium to attach to surfaces and colonize different environments while swimming and swarming facilitate the dissemination of *P. aeruginosa* within the host circulation [121]. Using the pertinent protocols, we demonstrated that the *surE* Tn-mutant, exhibits impaired swimming, swarming and twitching motility compared to the wild-type strain (**Figure 16 A-F**).

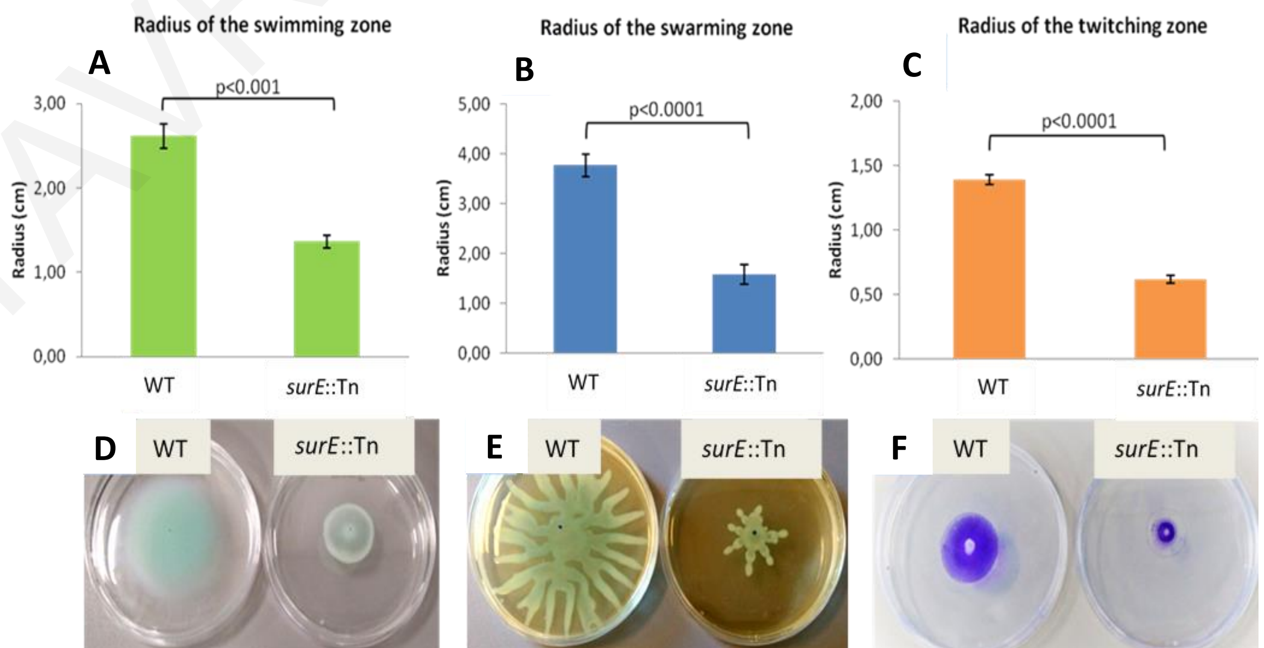

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Figure 16. Motility phenotypes of the *surE* mutant. The *surE* mutant exhibits defective motilities compared to the wild-type PA14. (**A-C**) The radius of the swimming, swarming and twitching zone respectively, in the *surE* mutant (on the right) versus the wild-type strain (on the left). (**D-F**) Photos of the phenotypes of the *surE* mutant (on the right) versus the wild-type strain (on the left) in the three types of motilities: (**D**) swimming motility, (**E**) swarming and (**F**) twitching motility.

4.7 *surE* regulates the expression of T3SS

As mentioned above, T3SS has a significant role in cytotoxicity and acute infections. For this reason, we expanded our study in more T3SS genes and we studied their expression in the *surE* Tn-mutant under Ca^{2+} limiting conditions known to activate the T3SS (5mM EGTA). We found that, compared to the wild-type strain, the *surE* mutant, has significantly lower expression of key T3SS genes, including two regulatory (*exsA*, *exsC*) and two effector proteins (*exoT*, *exoU*) (**Figure 17**).

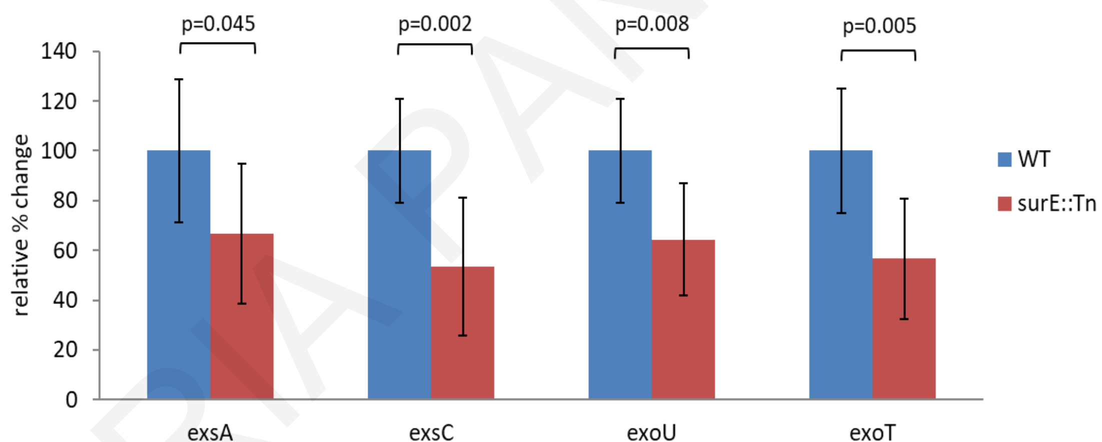

Figure 17. T3SS expression in the *surE* mutant. The *surE* gene is required for the expression of T3SS genes. The graph shows the relative % change in the expression of the T3SS genes *exsA*, *exsC*, *exoU*, and *exoT*, of the *surE* mutant compared to the wild-type strain.

4.8 *surE* controls the T6SS and Biofilm formation in *P. aeruginosa*

Although we were interested to identify metabolic genes that are important for acute infection, we were also curious to see what effect a mutation in such genes could cause in virulence factors that are more related to chronic infections. As mentioned in the introduction there is a reversible mechanism controlling the transition from the acute to the chronic state of infection, thus we assumed that the *surE* mutant would exhibit increased expression in

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virulence factors associated with chronic infection. Indeed, the *surE* mutant exhibits increased biofilm formation compared to the wild-type PA14 (**Figure 18**).

Additionally, the expression of the T6SS genes *clpV1*, *hcp1*, and *vgrG1*, was significantly increased in the *surE* mutant compared to the wild-type PA14 (**Figure 19**).

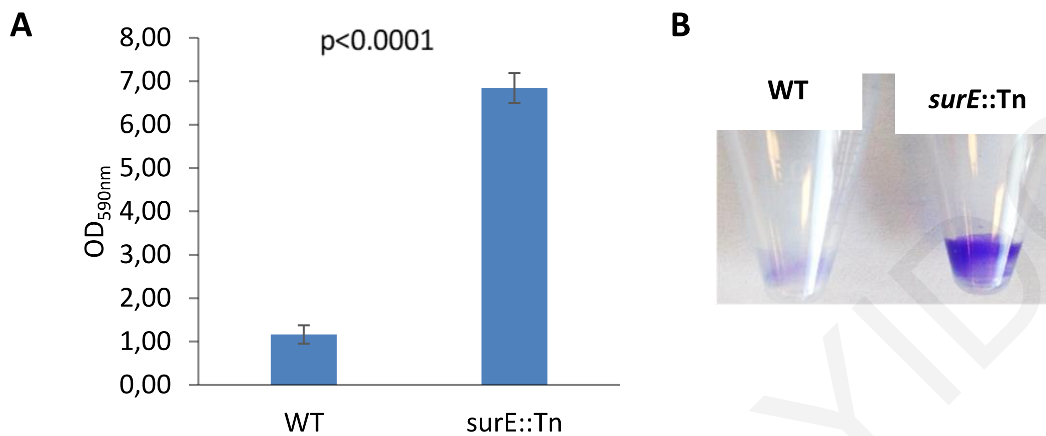

Figure 18. Biofilm formation in the *surE* mutant. (**A**) Biofilm formation (indicated by absorbance at 590 nm) for the *surE* mutant and the wild-type PA14. (**B**) Photo showing the crystal violet staining of the biofilm in the *surE* mutant and in the wild-type PA14.

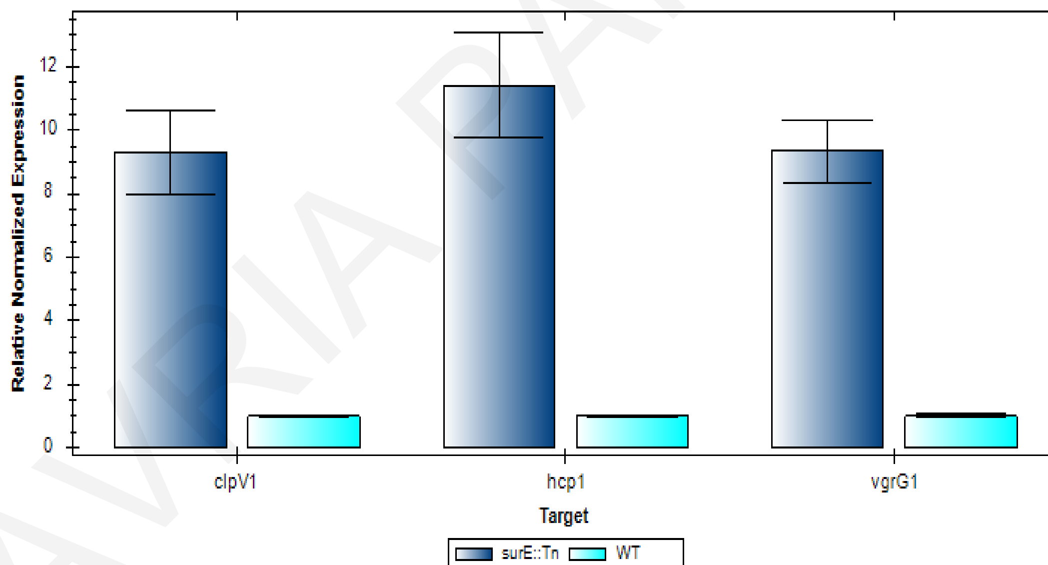

Figure 19. T6SS expression in the *surE* mutant. Relative normalized expression of three T6SS genes in the *surE* mutant and in the wild-type strain. The results are based on biological triplicates for each strain. The experiment repeated twice with different samples.

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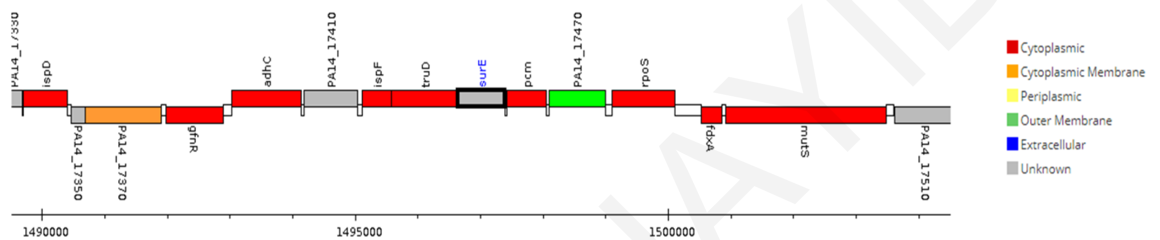
4.9 Steps for the complementation of the *surE* gene in the *surE* mutant

4.9.1 Validation of the transposon insertion in the *surE* mutant

To confirm, that the defective phenotypes of the *surE* Tn-mutant were linked only to the *surE* gene, we decided to complement the mutated gene with a plasmid. This was important because we observed that the *surE* gene is in the middle of a predicted operon (**Figure 20 A**) which, was consisted of 5 genes in total (*ispF, truD*, *surE*, *pcm* and PA14_17470). Regarding this, it was possible to have false phenotypes due to polar effects on downstream genes. Therefore, before the complementation process, we made some tests. First, we verified by PCR, the existence of the transposon in the *surE* mutant (**Figure 20 B, C**).

A *Pseudomonas aeruginosa* UCBPP-PA14, PA14_17450 (surE)



B

SEARCH RESULT

Records Per Page: 5 Sort By: Mutant ID Ascending Page Number: 1

Show Additional Information Output Format: By geneID Tabular

Results 1 to 1 of 1 genes Request Checked Mutants New Search

Locus Name	Gene ID	PAO1 Homolog	Gene Name	Genome Sequence ID	Strand	Gene Start	Gene Stop	Gene Length
PA14_17450	GID3167	PA3625	surE	2	+	1496636	1497385	750

Gene Description: putative stationary-phase survival protein SurE — Gene Status: ACTIVE

	Mutant ID	Blast Bit Score	Bases From Gene Start	Genetic Background	Transposon	Genome Location	PA14 NR Set Plate Position	NR Set Comments
	795	260.0	105	PA14	PhoA	1496741		
	27932	521.0	88	PA14	MrT7	1496724	PAMr_nr_mas_03_2:D9	

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*Note: PA14NR set mutants in red highlight.

```
>MAR2xT7 Transposon Sequence
TAACAGGTTGGCTGATAAGTCCCCGGTCTCTAGACCCTATAGTGAGTCGTATTACGCGGCCGCCGCGGCC
GGGAAGCCGATCTCGGCTTGAACGAATTGTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTT
CTTCCCGTATGCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTAGAT
CACATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGGTGGCAATGCCCTGCCTC
CGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCTCACTACGCGGCTGCTCAAACCTGGGCAGAA
CGTAAGCCGCGAGAGCGCCAACAACCGCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACG
GAGCAAGTTCCCGAGGTAATCGGAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACGA
CCGAAAAGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTACATGTGCGAATGATGC
CCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTGCGTAA
CATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGG
CATAGACTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGT
TCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTACGAACCGAACAGG
CTTATGTCAACTGGGTTCGTGCCTTCATCCGTTTCCACGGTGTGCGTCCATGGTAGCTAGCCGCGGGACC
GAGATAGGGTTGAGTGTTGTTCCAGTTTGTAATACGACTCACTATAGGGGTCTAGAGACCGGGGACTTAT
CAGCCAACCTGTTA
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C

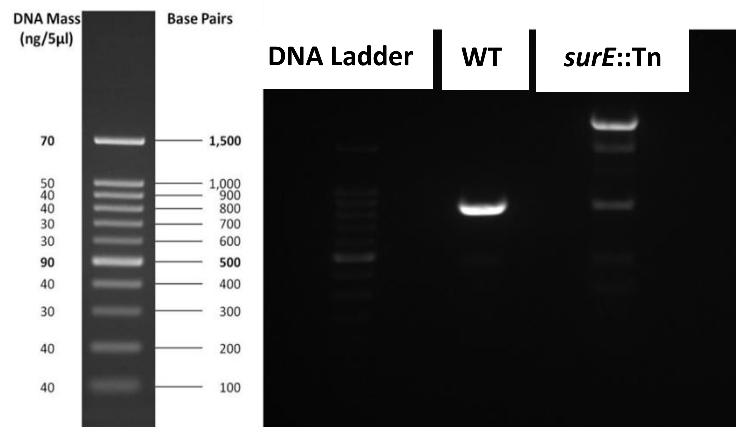

Figure 20. Validation of the *surE* transposon insertion by PCR. (**A**) The *surE* gene is in the middle of a predicted operon which is consisted of 5 genes in total (*ispF, truD*, *surE*, *pcm* and PA14_17470). (**B**) The sequence of the transposon inserted in the *surE* gene consisted of 994 nucleotides. (**C**) PCR for the *surE* gene in the wild-type strain and in the *surE* mutant. The sequence size of the *surE* gene in the wild-type PA14 is 750 nucleotides, while in the *surE* mutant is about 1750 nucleotides due to the insertion of the transposon.

4.9.2 Phenotypes of other genes located in the *surE*-operon

Next, we tested the swimming motility of the respective transposon mutants of the other genes located in the *surE* operon. We previously observed an extreme phenotype of the *surE* Tn-mutant in the swimming motility compared to the wild-type strain, thus any defects in the motility of the other operon genes would also connect them with this phenotype. Since no defect was observed in the swimming motility of these mutants (**Figure 21**), we assumed that they are not connected with the *surE* phenotypes.

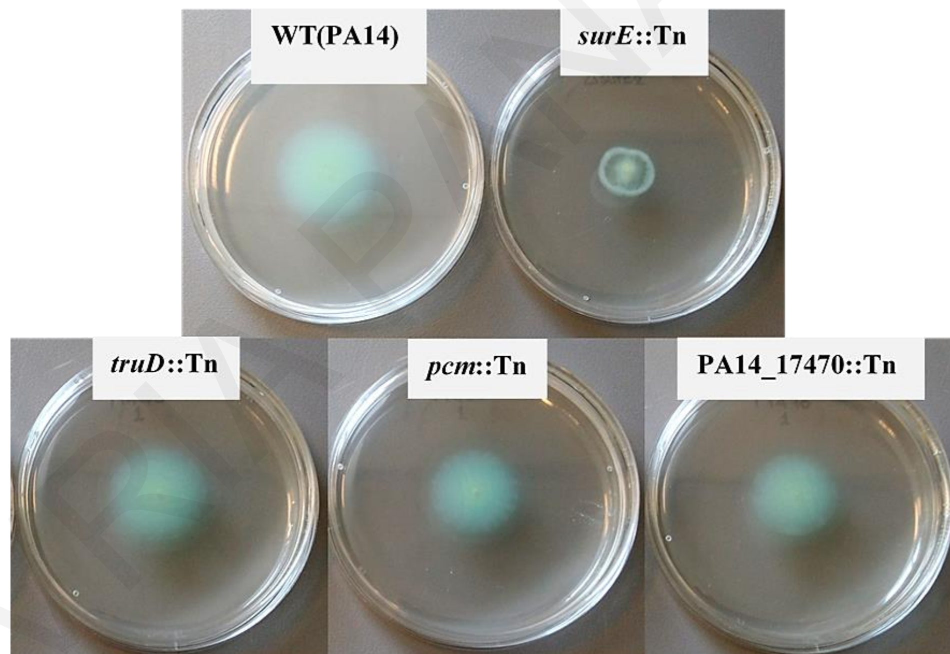

Figure 21. Swimming motility of *surE* operon gene members. Swimming motility of the wild-type PA14, the *surE* mutant and other gene mutants located in the same operon. Only the *surE* mutant exhibits defective swimming.

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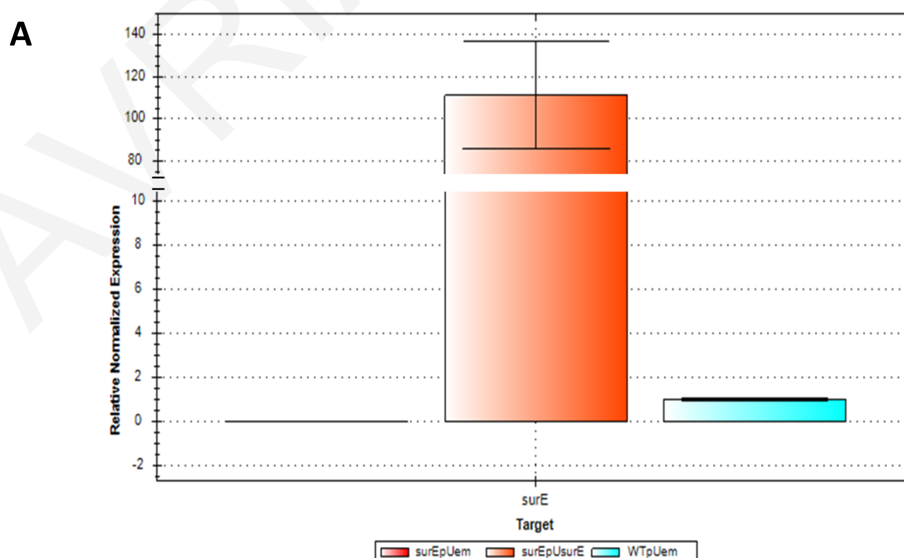
4.9.3 Cloning results

4.9.3.1 Restore of the *surE* expression in the *surE*-complemented strains

After following the procedure described in the methodology, we verified the complementation of the *surE* gene in the mutated strain by RT-qPCR. We examined the expression levels of *surE* in the following strains:

i. a mutated *surE* strain carrying the empty vector pUCP20
ii. a mutated *surE* strain carrying the vector pUCP20 containing the *surE* gene
iii. a wild-type PA14 carrying the empty vector pUCP20
iv. a mutated *surE* strain carrying the empty vector pDN19
v. a mutated *surE* strain carrying the vector pDN19 containing the *surE* gene
vi. a wild-type PA14 carrying the empty vector pDN19

We observed that the mutated *surE* strain carrying the empty vector pUCP20 (**Figure 22 A**), or the empty vector pDN19 (**Figure 22 B**), has zero expression of the *surE* gene. On the other hand, in the mutated *surE* strain carrying the high-copy vector pUCP20 containing the *surE* gene, the expression of the *surE* gene was upregulated at approximately 100-fold, compared to the wild-type PA14 carrying the empty vector pUCP20 (**Figure 22 A**). Additionally, the *surE* gene was found 14-fold more expressed in the mutated *surE* strain carrying the low-copy vector pDN19 containing the *surE* gene, compared to the wild-type PA14 carrying the empty vector pDN19 (**Figure 22 B**). These results indicate that there was a successful insertion of the *surE* gene into the two plasmids (pUCP20 and pDN19) and a successful transfer of the plasmids to the respective strains.



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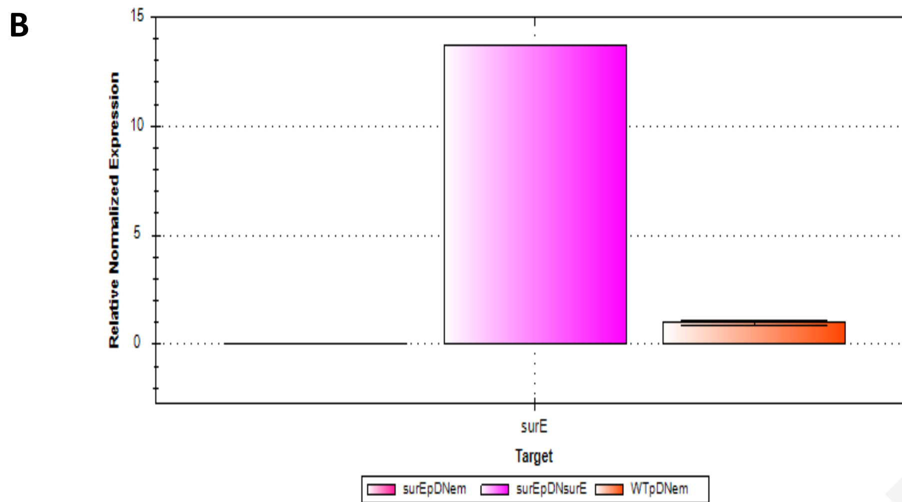

Figure 22. Validation of the *surE* expression in the *surE*-complemented strains. (A) Complementation results using the high-copy vector pUCP20. **(B)** Complementation results using the low-copy vector pDN19.

The graphs show the normalized expression of the *surE* gene, first in the mutated *surE* strain carrying each one of the empty vectors, next in the mutated *surE* strain carrying each vector containing the *surE* gene and last in the wild-type PA14 carrying each one of the empty vectors.

4.9.3.2 Phenotypes of the *surE*-complemented strains.

To see if there is at least partial rescue of the phenotypes observed by the *surE* mutant, in the *surE*-complemented mutants, we examined the swarming and twitching motility in the *surE*-complemented strains. Unfortunately, none of the two types of motilities was recovered in the *surE*-complemented mutants. Instead they exhibited defective swarming and twitching similarly to the *surE* mutant (**Figure 23 A-C**). In addition, the virulence of the *surE*-complemented strains was assessed in the feeding assay to see if there is a recover of virulence. However, the survival kinetics of the flies infected with the *surE*-complemented strains was similar to those infected with the *surE* mutant.

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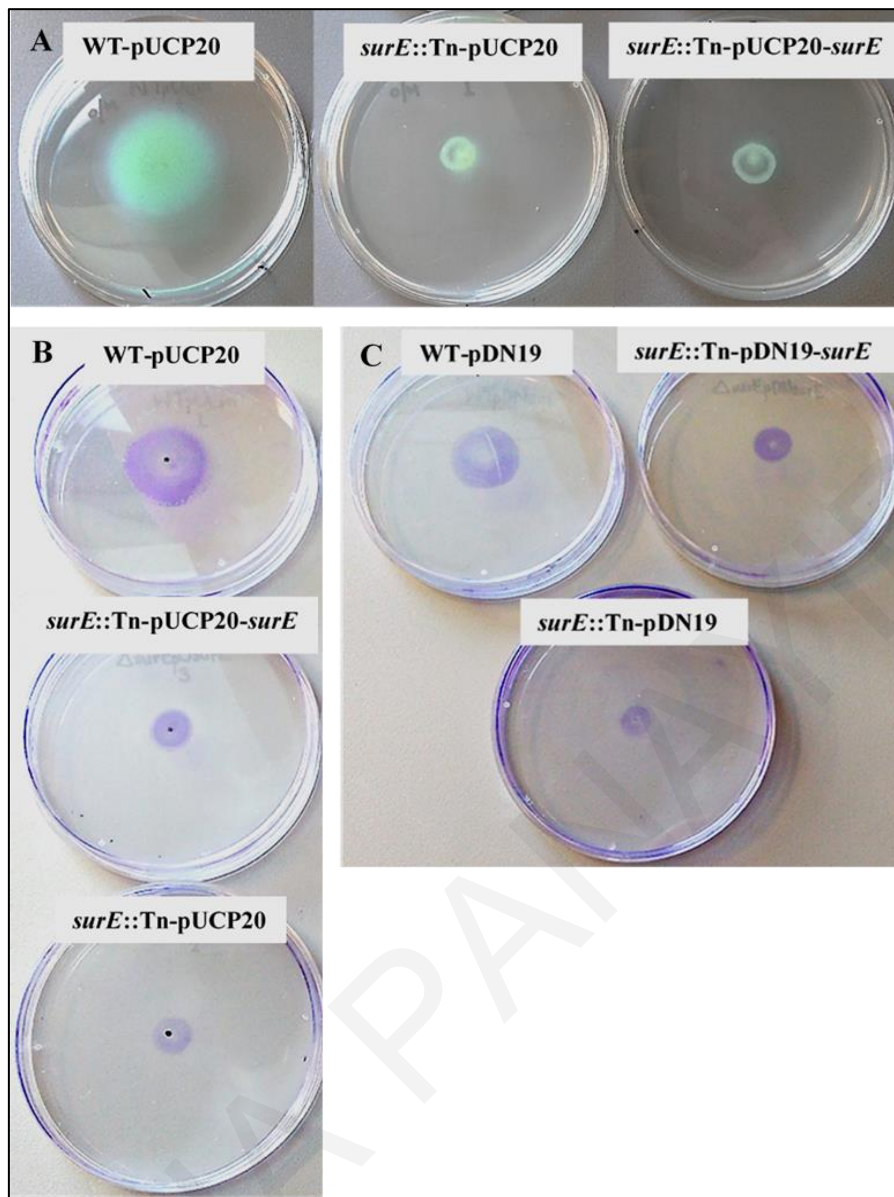

Figure 23. Motility phenotypes of the *surE*-complemented strains. (A) Swarming motility of the wild-type and the *surE* mutant carrying the empty pUCP20 vector and of the *surE* mutant carrying the pUCP20 vector with the *surE* gene. **(B)** Twitching motility of the wild-type and the *surE* mutant carrying the empty pUCP20 vector and of the *surE* mutant carrying the pUCP20 vector with the *surE* gene. **(C)** Twitching motility of the wild-type and the *surE* mutant carrying the empty pDN19 vector and of the *surE* mutant carrying the pDN19 vector with the *surE* gene. Each condition was in triplicates.

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CHAPTER 5

EXTENSION OF THE MAIN PROJECT

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5.1 Extension of the main project - Identifying common virulence-related metabolic pathways between pathogenic *P. aeruginosa* strains

In recent years there is a significant contribution of transcriptomic and proteomic techniques for the characterization of bacterial gene expression under different environmental conditions [101]. Comparative genomic approaches can also provide valuable information based on sequence homology of proteins with known functions (e.g. virulence factors) in different bacterial strains [101].

Our goal was to identify common virulence-related metabolic pathways, among pathogenic *P. aeruginosa* strains that could serve for drug target identification. *P. aeruginosa* pathogenicity has been characterized as context-dependent, which means that genes required for pathogenicity in one strain may not necessarily contribute to virulence in other strains. Through our collaboration with Dr. Vasilis Promponas group, we found no correlation between pathogenicity and gene content in 30 *Pseudomonas* strains. For this reason, we hypothesized that differential expression of virulence-related genes (including metabolic genes) could be able to explain differences in the pathogenicity among *P. aeruginosa* strains. Thus, we used functional transcriptomics of highly and lowly pathogenic *P. aeruginosa* strains to identify conserved core-metabolism modules related to virulence. The presence of common virulence-related metabolic modules in various *P. aeruginosa* strains may indicate that genes belonging in these modules are more likely to be essential for the pathogenicity and thus could be ideal candidates for drug target identification.

5.1.1 Assessment of the pathogenicity of 30 fully sequenced *Pseudomonas* strains in *Drosophila melanogaster*.

To correlate the pathogenicity of 30 fully sequenced *Pseudomonas* strains (18 *P. aeruginosa* and 12 other *Pseudomonas* strains; **Table 5**), we examined their virulence in *Drosophila melanogaster*, using an oral and a wound infection assay that impose two distinct types of acute infection (**Figure 24 A, B**). Kaplan-Meier survival analysis with a log-rank test and pairwise comparison over strata was used to analyze the results of fly survival after the infection. Even though it is usually intuitive to group bacterial strains based on visual inspection of their survival curves, this procedure is subjective. To objectively partition the strains under study based on their pathogenicity, the 30 bacterial strains were classified in three groups (low, medium and high), depending on the severity of their virulence phenotype (**Figure 24 A, B** and **Table 5**), using hierarchical clustering (data not shown). We used the

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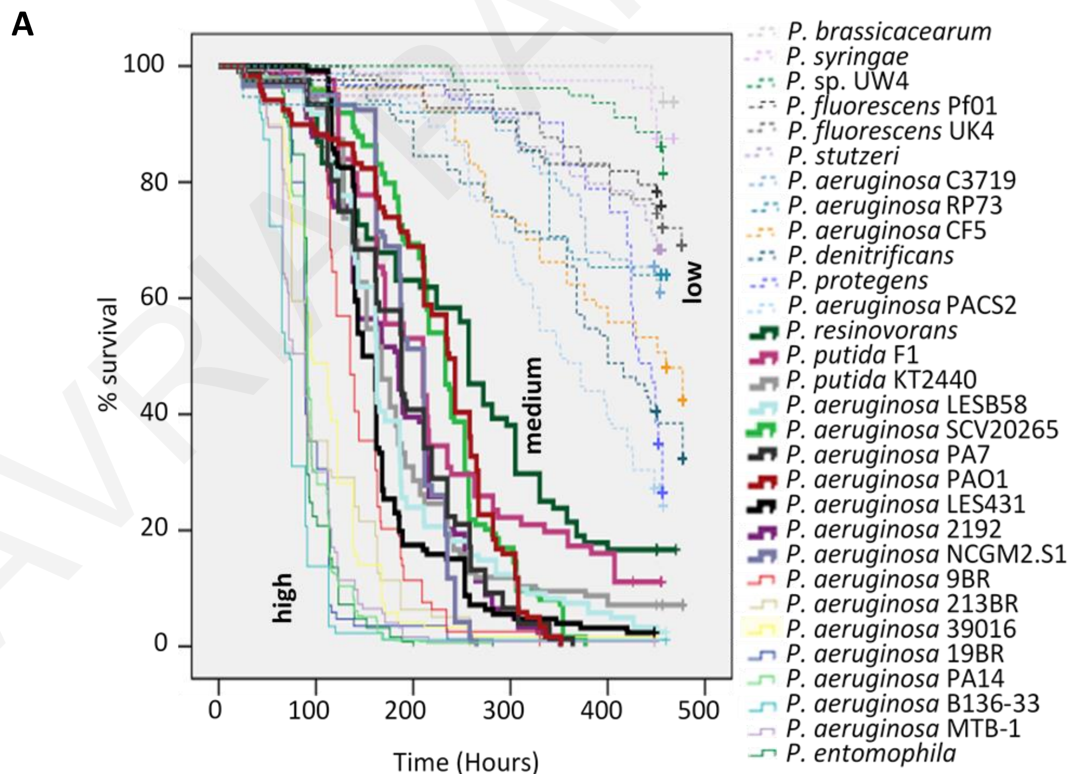
PA14 and CF5 strains to define the 'high' and 'low' virulence categories, respectively, in both assays, in accordance with published data [114, 122].

Non-*P. aeruginosa* strains tend to consistently group in the 'low' virulence cluster, with a notable exception of *P. entomophila* presence in the 'high' virulence cluster. *P. entomophila* is a known entomopathogenic bacterium, able to infect and kill insects, including *Drosophila* [123]. *P. aeruginosa* strains B136-33, MTB-1, PA14, 213BR, 19BR, 9BR and 39016, on the other hand, were consistently virulent, while strains CF5 and C3719 consistently low in virulence regardless of the infection assay. Some *P. aeruginosa* strains were less consistently grouped between assays, such as PAO1, which was grouped with the 'high' and 'medium' virulence cluster in the wound and oral infection assays respectively. To more rigorously select highly and lowly virulent strains we used a model of acute intranasal mouse lung infection examining the mortality rate of four of the highly (B136-33, MTB-1, PA14, 213BR) and the three most lowly (C3719, CF5, PACS2) in *Drosophila* virulence *P. aeruginosa* strains. We found that all but one (213BR) of the 7 tested strains retained their virulence potential in the acute mouse lung infection model (**Figure 24 C**).



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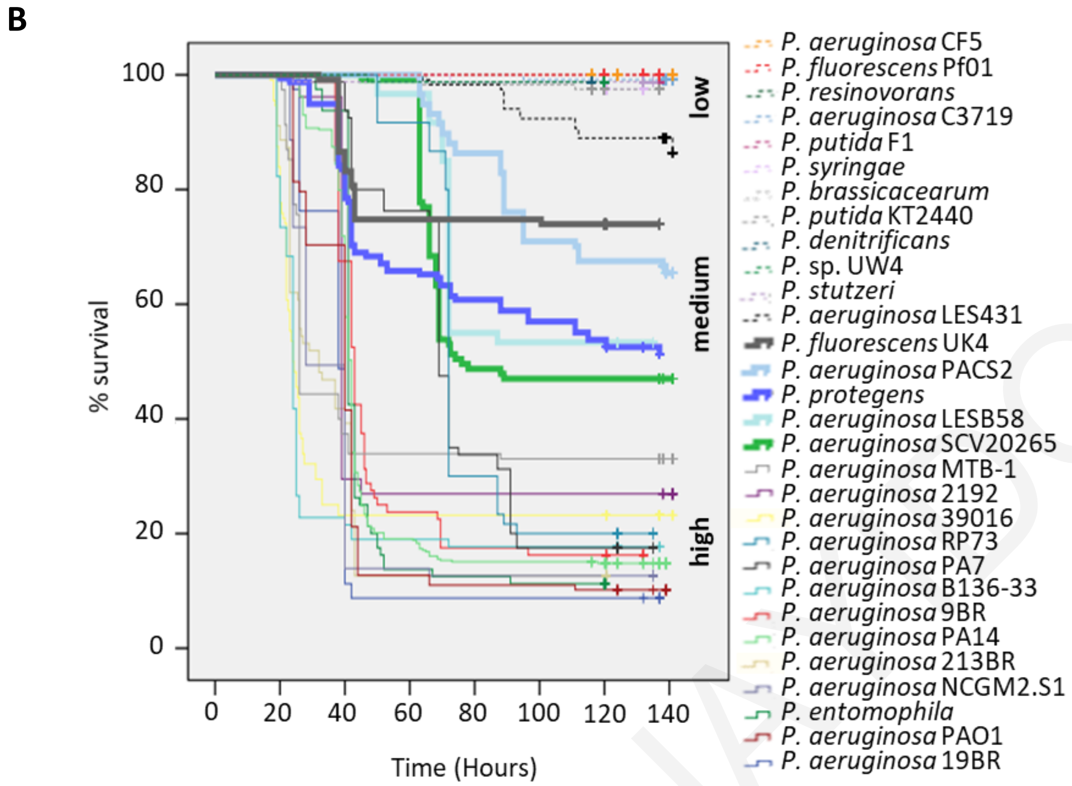

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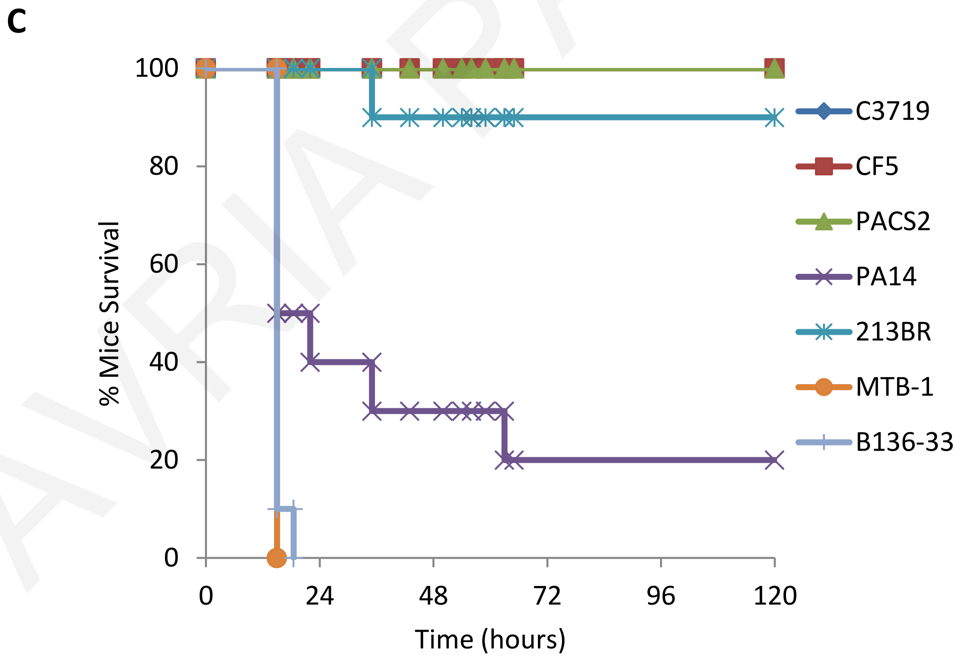

Figure 24. Comparative survival of *Pseudomonas* strains upon oral and wound infection in flies and lung infection in mice. Three groups are distinguished, each representing species that are either high, medium or low in virulence, in the fly (**A**) oral and (**B**) wound infection. Thin lines represent high virulent strains, thick lines represent medium virulent strains and dashed lines represent low virulent strains. Time is measured in hours.

(**C**) Mouse survival (%) after intranasal mice infection with 3 low and 4 highly virulent *P. aeruginosa* strains. Twenty-microliter aliquots of a bacterial solution containing $2x10^7$ bacteria were administered intranasally to each mouse. Infected mice were monitored for 5 days (n=10).

Table 5. Pathogenicity ranking according to oral and wound infection in flies. Pathogenicity ranking according to oral and wound infection in flies. *Pseudomonas* species used in this study include 18 *P. aeruginosa* and 12 non-*P. aeruginosa* fully sequenced strains. The red color corresponds to the highly virulent strains, the black to those of medium virulence and the blue to lowly virulent, in the feeding and/or the pricking assay (last two columns).

#	SPECIES	STRAIN	SOURCE	Laboratories	Feeding Assay	Pricking Assay
					LT50% (hours)	
1	*P. aeruginosa*	B136-33	Chuang *et al.*, 2014 [124]	Prof. Cheng-Hsun Chiu	61,02	22,60
2	*P. aeruginosa*	213BR	Boyle *et al.*, 2012 [125]	Prof. Bob Hancock	83,00	31,79
3	*P. aeruginosa*	19BR	Boyle *et al.*, 2012 [125]	Prof. Bob Hancock	85,13	35,58
4	*P. aeruginosa*	9BR	Boyle *et al.*, 2012 [125]	Prof. Bob Hancock	129,10	40,26
5	*P. aeruginosa*	MTB-1	Ohtsubo *et al.*, 2014 [126]	RIKEN Bioresource Center (RIKEN BRC)	86,00	29,62
6	*P. aeruginosa*	PA14	Lee *et al.*, 2006 [114]	Prof. Yiorgos Apidianakis	86,12	38,13
7	*P. aeruginosa*	39016	Steward *et al.*, 2011 [127]	Prof. Craig Winstanley	101,90	27,58
8	*P. aeruginosa*	LES431	Jeukens *et al.*, 2014 [128]	Prof. Craig Winstanley	150,68	**89,0%
9	*P. aeruginosa*	PA7	Roy *et al.*, 2010 [129]	Prof. Stephen Lory	166,53	71,28
10	*P. aeruginosa*	LESB58	Winstanley *et al.*, 2009 [130]	Prof. Craig Winstanley	168,72	**53,8%
11	*P. aeruginosa*	2192	Mathee *et al.*, 2008 [131]	Prof. Stephen Lory	175,33	35,45
12	*P. aeruginosa*	NCGM2.S1	Miyoshi-Akiyama *et al.*, 2011 [132]	Dr. Toru Miyoshi-Akiyama	187,06	31,98
13	*P. aeruginosa*	SCV 20265	Eckweiler *et al.*, 2014 [133]	Prof. Susanne Häussler	222,36	**64,2%
14	*P. aeruginosa*	PAO1	Stover *et al.*, 2000 [134]	Prof. Stephen Lory	224,35	32,95
15	*P. aeruginosa*	PACS2	Mathee *et al.*, 2008 [131]	Prof. Stephen Lory	350,49	**66,0%
16	*P. aeruginosa*	CF5	Broadinstitute.org, 2013	Prof. Yiorgos Apidianakis	388,88	**100%
17	*P. aeruginosa*	C3719	Mathee *et al.*, 2008 [131]	Prof. Stephen Lory	*62,8%	**99,2%
18	*P. aeruginosa*	RP73	Jeukens *et al.*, 2013 [135]	Dr. Alessandra Bragonzi	*63,9%	71,28

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19	*P. stutzeri*	ATCC 17588	Chen *et al.*, 2011 [136]	International Center for Microbial Ressources - French Collection for Plant-associated Bacteria (CIRM-CFBP)	*69,3%	**98,8%
20	*P. fluorescens*	UK4	Dueholm *et al.*, 2014 [137]	Prof. Morten Simonsen Dueholm & Prof. Halkjær Nielsen	*72,3%	**73,9%
21	*P. fluorescens*	Pf01	Silby *et al.*, 2009 [138]	Prof. Stuart B. Levy	*77,0%	**100%
22	*P. sp. UW4*	UW4	Duan *et al.*, 2013 [139]	Prof. Bernard R. Glick	*79,5%	**98,8%
23	*P. syringae*	DC3000	Buell *et al.*, 2003 [140]	Prof. Víctor Flors Herrero	*91,4%	**96,5%
24	*P. brassicacearum*	NFM421	Ortet *et al.*, 2011 [141]	International Center for Microbial Ressources - French Collection for Plant-associated Bacteria (CIRM-CFBP)	*100%	**100%
25	*P. entomophila*	L48	Vodovar *et al.*, 2006 [123]	Prof. Yiorgos Apidianakis	85,88	44,32
26	*P. putida*	KT2440	Nelson *et al.*, 2002 [142]	Prof. Víctor de Lorenzo Prieto	152,33	**97,3%
27	*P. putida*	F1	Gibson *et al.*, 1968 [143]	Prof. Morten Simonsen Dueholm & Prof. Halkjær Nielsen	190,07	**99,2%
28	*P. resinovorans*	CA10	Shintani *et al.*, 2013 [144]	RIKEN Bioresource Center (RIKEN BRC)	222,89	**97,8%
29	*P. denitrificans*	ATCC 13867	Ainala *et al.*, 2013 [145]	Prof. Sunghoon Park	390,43	**98,8%
30	*P. protegens*	Pf-5	Paulsen *et al.*, 2005 [146]	Prof. Morten Simonsen Dueholm & Prof. Halkjær Nielsen	426,38	**60,6%
	*: % survival at endpoint (450 hours) **: % survival at endpoint (120 hours) Red: high / Blue: low / Black: medium					

***Acknowledgments**: We thank our collaborator Dr. Theodoulakis Christofi, former lab member of Prof. Yiorgos Apidianakis lab, for collecting all the above strains and for performing most of the above experiments as well as the analysis of the results. We also thank all the above laboratories for providing the strains.*

5.1.2 Assessment of the pathogenic profiles of highly and lowly pathogenic *P. aeruginosa* strains against transcriptomic data

In collaboration with Dr. Promponas group, we investigated the differences in pathogenicity among highly and lowly pathogenic *P. aeruginosa* strains, compared to their transcriptomic profiles. To identify differentially expressed genes relating to pathogenicity we performed a transcriptome analysis of the 6 *P. aeruginosa* strains validated in mice as 'high' (B136-33, MTB-1, PA14) or 'low' (C3719, CF5, PACS2) in virulence. RNA was extracted from bacteria grown at mid-exponential and early stationary phase (optical density OD_{600nm} 1 and

3, respectively). We selected genes differentially regulated in the same direction (either over- or under-expressed) between all pairs of 'high' versus 'low' in virulence strains. Nine of them were differentially expressed at both growth phases (8 of which were overexpressed in highly virulent strains) (**Figure 25**). At OD_{600nm} 1, 15 genes were up, and 6 genes were down in all 3 highly versus all 3 lowly pathogenic species (**Figure 25**). At OD_{600nm} 3, 52 were up and 2 genes were down in all 3 highly versus all 3 lowly pathogenic species (**Figure 25**). Overall 20 VFs (most of them being quorum sensing and T3SS related), 11 metabolic genes, 5 transcriptional regulators and several hypothetical proteins were found differentially expressed. The fact that several metabolic genes appear among the consistently differentially expressed genes begs the question of the broader contribution of metabolism in *P. aeruginosa* virulence.

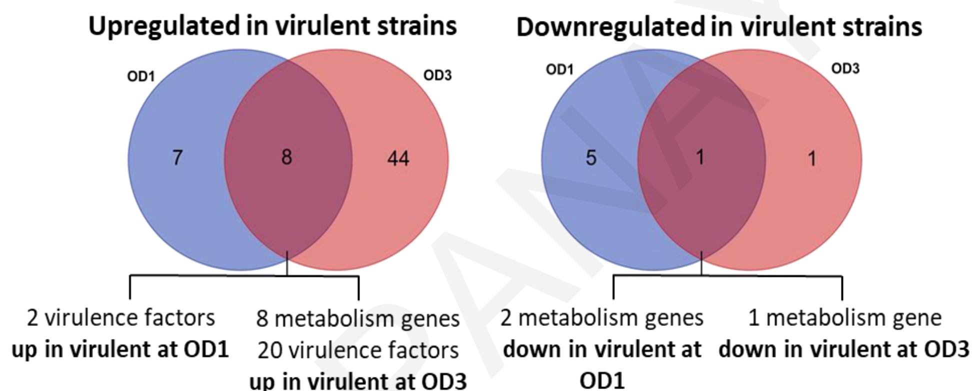

Figure 25. Genes differentially expressed between 3 lowly and 3 highly virulent *P. aeruginosa* strains. VENN diagram of transcriptomics analysis of the 66 differentially expressed genes using http://bioinformatics.psb.ugent.be/webtools/Venn/. At OD_{600nm} 1, 15 genes were up, and 6 genes were down in all 3 highly versus all 3 lowly pathogenic species. At OD_{600nm} 3, 52 were up and 2 genes were down in all 3 highly versus all 3 lowly pathogenic species.

Acknowledgments: *Bacterial RNA sequencing was performed at the BSRC Al. Fleming Genomics Facility. We thank Dr. Vaggelis Harokopos for the NGS experiments and Dr. Martin Reczko for initial bioinformatic analyses.*

5.1.3 Identification of metabolism gene patterns related to virulence

To pinpoint metabolic gene expression patterns important for *P. aeruginosa* virulence, we assessed the differential expression of the 78 core-metabolism genes required for the full virulence of *P. aeruginosa* strain PA14 in the 3 selected high versus the 3 selected low in virulence *P. aeruginosa* strains. Close inspection of differential gene expression in all

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possible 'high' versus 'low' strain comparisons reveals that there is no consistent differential gene expression at any of the bacterial growth phases. That is, 45 of the 78 functionally important metabolic genes do not exhibit any differential expression among any of the 18 comparisons performed. The remaining 33, for which statistically significant up- or down-regulation was observed in at least one tested case, are mostly differentially expressed in the minority of 'high' versus 'low' virulence comparisons.

However, using the BioCyc Pathway/Genome Database Collection we identified 107 differentially expressed metabolic pathways containing genes differentially expressed between at least one highly (PA14, MTB-1, B136-33) versus all 3 lowly (C3719, CF5, PACS2) virulent strains and pathways containing one or more of the 78 functionally validated genes. Overlapping these pathways, we find 8 of them containing genes upregulated in all virulent strains against all low in virulence strains and at least one gene that compromises virulence when mutated (**Figure 26 A**). These common pathways are related to (i) the 4-hydroxyl-phenylacetate degradation and succinate production, (ii) the glutamine biosynthesis from glutamic acid, (iii) the shikimate and chorismate biosynthesis from D-erythrose 4-phosphate, (iv) the branched-chain amino acid biosynthesis of leucine, (v) the 2,5- and 3,5-xylenol degradation to citramalate, and (vi) the beta-oxidation of fatty acids (**Figure 26 B**). These data strongly support the idea that differences in virulence among *P. aeruginosa* strains are arising from differential gene expression of genes belonging in specific core metabolism pathways.

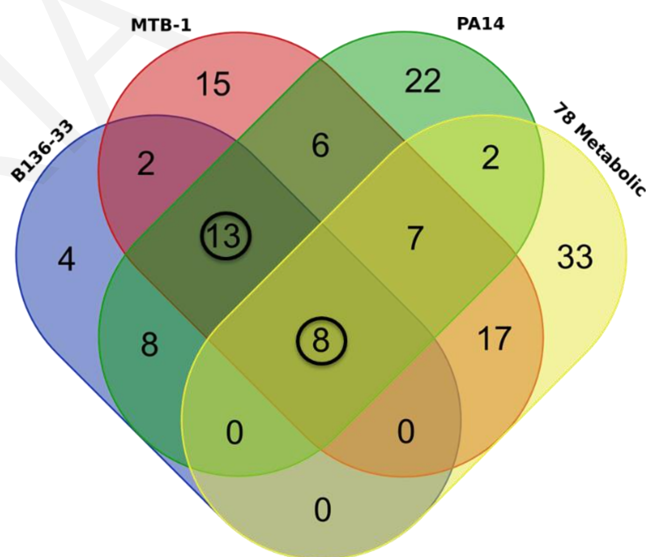

Figure 26. Overlap among 4 metabolic gene pathway groups. Three expression-based metabolic pathway groups arise from differentially expressed genes comparing each of the 3 high (B136-33, MTB-1 or PA14) with all 3 low (CF5, C3719 and PCS2) in virulence strains, while the 4th functionality-based metabolic pathway group arises from the 78 genes

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functionally important for full virulence in flies. Out of totally 137 metabolic pathways assessed for overlap based on BioCyc Database Collection (https://biocyc.org) 21 were common to the 3 expression-based metabolic pathway groups, while 8 of them were also functionally important for virulence. We circle the numbers of the 8 functionality-based plus 13 more, totaling 21 common expression-based metabolic pathways.

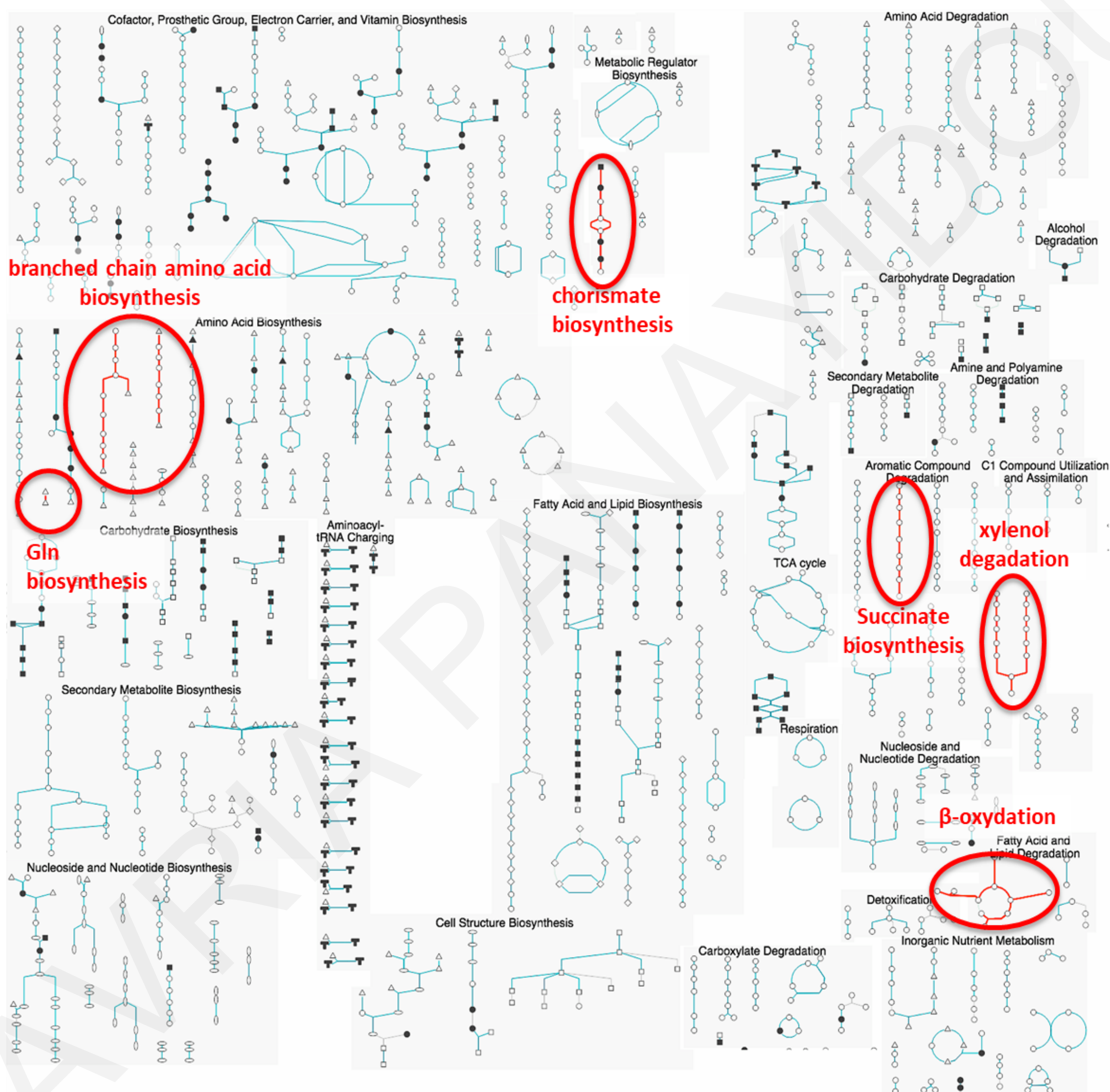

Figure 27. Common metabolic pathways implicated in *P. aeruginosa* virulence. The common pathways are related to: (i) the 4-hydroxyl-phenylacetate degradation and succinate production, (ii) the glutamine biosynthesis from glutamic acid, (iii) the shikimate and chorismate biosynthesis from D-erythrose 4-phosphate, (iv) the branched chain amino acid biosynthesis of leucine, (v) the 2,5- and 3,5-xylenol degradation to citramalate, and (vi) the beta-oxidation of fatty acids.

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CHAPTER 6

DISCUSSION

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6. Discussion

Common antibiotics usually kill or inhibit the growth of sensitive strains and shift the competitive balance in favor of drug-resistant strains [102, 103]. This causes the prevalence of drug-resistant strains and usually leads to the exacerbation of the infection and spread of antibiotic-resistance [102, 103]. In recent years, the usage of virulence-factors as targets for new therapeutic drugs emerged as an alternative approach that could mitigate the development and spread of antibiotic resistance [104]. Our long-term goal is to identify virulence-related metabolic genes that could serve as novel targets for drug development against *P. aeruginosa*. In this study, we investigated the contribution of *P. aeruginosa* metabolism in virulence. Considering the role of metabolism in bacterial growth, we focused only on virulence-related metabolic genes that are not essential for growth.

Next, we correlated the transcriptome of 3 low and 3 high in virulence *P. aeruginosa* strains to identify differences in virulence-related genes, including core-metabolism genes, that could distinguish strains with different pathogenic potential. This approach can be useful for identifying core-metabolism genes as candidate targets for drug development against pathogenic *P. aeruginosa* strains.

6.1 Investigating the role of *P. aeruginosa* metabolism in virulence

To reach our goal, we initially investigated the role of all the PA14 metabolic genes in virulence using the PA14 Transposon Insertion Mutant Library of the Harvard Medical School. By assessing 553 metabolic and 95 non-metabolic gene mutants of the *P. aeruginosa* strain PA14 for virulence in *Drosophila melanogaster*, we found 16.5% of the metabolic and 8.5% of the non-metabolic genes to be important for full virulence. For the screen, wild-type female flies 3-7 days old were orally infected with PA14 strains diluted in a sucrose solution. In addition, male wild-type flies, grown together with the female flies, were wound infected with a needle dipped in a bacterial solution containing one PA14 strain at a time. Depending on the infectious agent differences can be noted between male and female flies regarding their susceptibility to bacterial infection [78]. However, no qualitative differences are usually expected between sexes. For example, male and female Oregon R flies clear *P. aeruginosa* infection at the same rate and shed the same number of *P. aeruginosa* CFUs [147].

Our Tn-mutant screen identified several metabolic genes necessary for full virulence in flies. However, the defective phenotype of the metabolic mutants, could not directly correlate the respective genes with virulence since some of these could primarily be involved in bacterial growth. Therefore, the growth-assessment of the selected PA14 metabolic mutants, in culture and in the host, was crucial in order to have a clear picture of which of the

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corresponding genes were virulence-related and not growth-essential. We found that 11.8% of the growth-independent metabolic genes (**Tables 2 & 3**) and 8.5% of the non-metabolic genes (**Table 1**, **Figure 9**) are directly linked to virulence. A summary diagram of the screen results is provided below (**Figure 28**).

Collectively, we found that there is an important contribution of *P. aeruginosa* metabolic genes in virulence, suggesting that metabolic genes could be used as candidates for drug target identification.

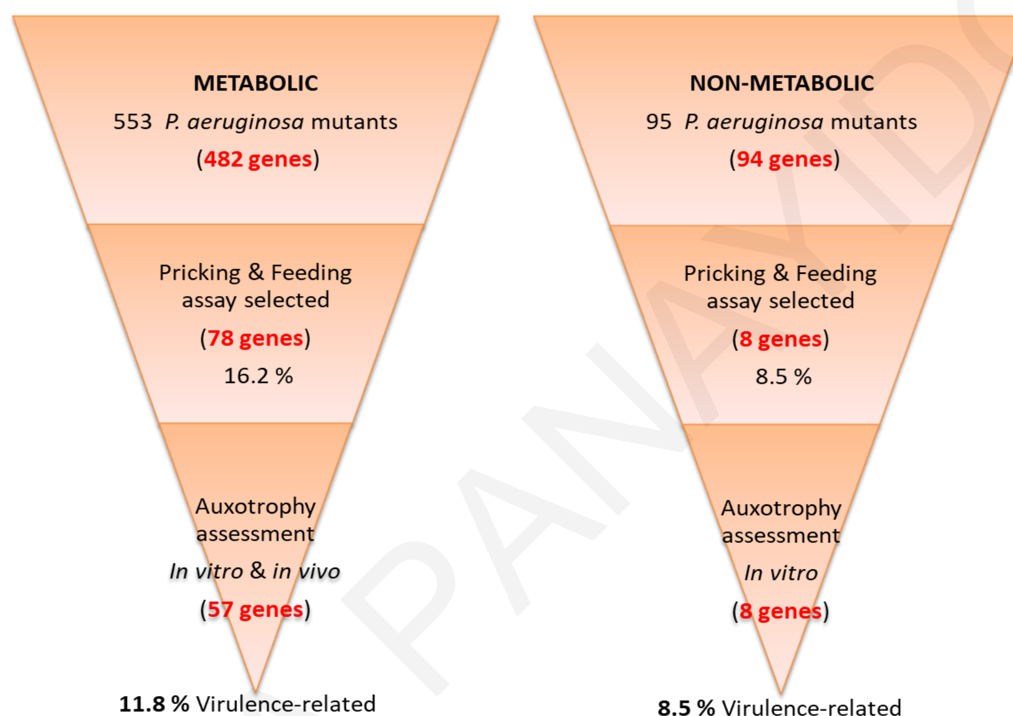

Figure 28. Diagram of the screen results and growth assessment. The screen of 553 *P. aeruginosa* metabolic Tn-mutants corresponding to 482 metabolic genes and of 95 randomly selected non-metabolic *P. aeruginosa* Tn-mutants corresponding to 94 non-metabolic genes, at two independent and in parallel performed infection assays (pricking and feeding), ended up with 16.2% of the metabolic and 8.5% of the non-metabolic *P. aeruginosa* genes, as necessary for full virulence. Further *in vitro* and *in vivo* examination of the growth capacity of the selected mutants revealed that there is an important contribution of *P. aeruginosa* metabolic genes in virulence since 11.8% of them are not important for growth in culture and/or in the host, thus must be related to virulence.

6.2 Growth-independent contribution of *P. aeruginosa* metabolic genes on virulence

We defined as virulence-related those genes that are necessary for the full virulence of *P. aeruginosa* independently of their effect on growth in minimal media or in the host. Thus, we included as growth-independent, mutants that are able to grow similarly to wild-type

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strain in (a) glucose minimal media, (b) glucose minimal media supplemented with fly extract and (c) the host according to the wound or the oral infection assay.

Based on our results, histidine mutants behaved as conditional prototrophs since, although unable to grow in glucose minimal medium, they could grow efficiently in glucose minimal medium supplemented with fly extract and were also able to colonize the flies similarly to the wild-type *P. aeruginosa* strain (**Table 3, Group A**). The virulence attenuation of these mutants is more likely due to defects in virulence rather than to growth defects. This is further supported by the fact that *hisD* mutant had low expression levels of the *exoT* gene, encoding an important effector protein of the T3SS (**Figure 15 A**). On the other hand, most of the purine mutants were considered as conditional prototrophs for being able to grow in glucose minimal medium that contained fly extract, however, these were unable to colonize flies to the same extent as the wild-type strain (**Table 3, Group B**). This suggests that although enough nutrients for growth exist in the host tissues, bacteria are unable to acquire them, most probably due to defects in virulence factors required for the initial steps of the infection. Consistent to that, *purD* mutant had significantly low levels of the type IV pilus gene *pilA* compared to the wild-type strain (**Figure 15 D**). Previous studies, showed that purine mutants including *purD*, exhibited reduced cytotoxicity and a modest reduction of biofilm formation [67]. Our studies also revealed that the *purN* mutant is a prototroph since it was able to grow in glucose minimal medium (**Table 2**). We assume that this occurs due to the overlap functions between purN and purT. Interestingly, purN and purT enzymes are implicated in the direct production of tetrahydrofolate (THF). THF but also FGAR synthesis by purN, occurs by the transfer of a formyl group from 10-formylTHF to GAR [reaction: *(6S)-10-formyltetrahydrofolate + N^1-(5-phospho-D-ribosyl)glycinamide = (6S)-5,6,7,8-tetrahydrofolate + H^+ + N^2-formyl-N^1-(5-phospho-D-ribosyl)glycinamide*, based on *https://www.uniprot.org/uniprot/A0A0H2Z6A5*]. FGAR can also be produced, if purT catalyzes the transfer of formate to GAR [reaction: *ATP + formate + N^1-(5-phospho-D-ribosyl)glycinamide = ADP + H^+ + N^2-formyl-N^1-(5-phospho-D-ribosyl)glycinamide + phosphate*, based on *https://www.uniprot.org/uniprot/Q02RM4*]. Thus, the *purN* mutation does not lead to growth reduction in glucose minimal medium, because de novo purine biosynthesis is not interrupted since FGAR production is replaced by the action of purT. However, *purN* mutation leads to virulence attenuation in both flies and mice. We also observed, that *purN* mutant is not able to colonize flies to the same extent as the wild-type PA14 and this defect must be related to virulence, because it also exhibits impaired swarming and twitching motility (**Figure 14 A, B**). In agreement with that, *purN* mutant had

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significantly low expression of the type IV pilus gene *pilA* as well as of the *exoT* gene, compared to the wild-type (**Figure 15 A, D**).

Similar phenotypes to that of the *purN* mutant were observed by the *folk* mutant, which indirectly leads to the production of THF. Interestingly, the *folk* mutant was assigned as prototroph because it was able to grow in glucose minimal medium as well as in the host (fly) (**Table 2**). However, it exhibited defective phenotypes in both swarming and twitching and low expression of *exoT* (**Figure 14 A, B; Figure 15 A**). The *metF* mutant is also important for the synthesis of 5-methyTHF, which is converted to THF during the synthesis of L-methionine from L-homocysteine by metH. The *metF* mutant failed to grow in glucose minimal medium like the wild-type, most probably due to methionine auxotrophy. However, it has the potential to grow in flies similarly to the wild-type strain since it grows in glucose minimal medium with 5% fly extract and colonizes the flies efficiently (**Table 3, Group A**). Most of the pyrimidine mutants were listed as auxotrophs because they were unable to grow efficiently in any of the growth assays (**Table 3, Group D**). The pyrimidine, as well as the purine biosynthesis pathway, are also important for DNA and RNA metabolisms [67]. Interestingly, recent work indicates that purine auxotrophy may be a common microbial strategy for adaptation in DNA-rich host tissues such as the CF lungs [148].

Importantly, we identified conditional prototrophs that although unable to grow efficiently in culture in any of the two assays, could colonize flies normally (**Table 3, Group C**); therefore we assumed that the corresponding genes are linked to virulence, irrespective of any growth defects.

To validate our approach, we assessed the virulence factor production in a subset of 13 metabolic gene mutants that were attenuated in virulence not only in flies but also in an acute murine lung infection assay (**Figure 13**). We found that all of them exhibit defects in at least one aspect of virulence. Among the 13 selected virulence-related PA14 metabolic genes, we pinpointed a novel gene, encoding *P. aeruginosa* putative stationary-phase survival protein-*surE*, which is a nucleotidase with a broad-spectrum phosphatase activity against nucleotide monophosphates. We observed that the *surE* mutant was deficient, in many virulence factors related to acute infection, including the three types of motility (swarming, swimming, and twitching) (**Figure 16**) and T3SS genes (**Figure 17**). Another 2 mutants were defective in most of the virulence factors: *miaA* and *rpoC* (**Table 6**). However, *miaA*, is a tRNA isopentenyl transferase, a tRNA modification enzyme important for the translation efficiency, while *rpoC* is the DNA-directed RNA polymerase beta subunit that has an important role in the transcription. We focused on *surE* because we wanted to study a novel

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gene that is more related to a metabolic function rather than to the translation and the transcription.

To confirm that the defective phenotypes of the *surE* Tn-mutant were linked only to the *surE* gene, we complemented the mutated gene, using different types of cloning vectors. Although the expression of the *surE* gene was recovered in the *surE* mutant after the complementation process, no clear rescue of the phenotypes was observed. We assume the lack of complementation is due to the production of a non-functional *surE* protein by the complemented strain or that the phenotypes of the *surE* mutant are caused by polar effects of the transposon on the expression of genes located downstream of the *surE* gene. Another possible explanation is that the *surE* mutant underwent additional mutations that caused its phenotypes.

Regarding the virulence phenotypes of the 13 virulence-related metabolic mutants, we sought to focus on mutants that have similar growth rates to the wild-type in the M9 medium. This is because even small differences in growth rates result in measurable differences in the size of different bacterial populations and this may affect the expression of virulence factors. Virulence-related metabolic genes that belong to biosynthetic pathways may produce metabolites as building blocks for effector proteins and other virulence factors. Mutations in such genes can also affect the levels of molecules that are important for virulence. For example, elevated levels of cyclic di-GMP suppress motility and stimulate biofilm formation [13]. On the other hand, virulence-related metabolic genes that do not belong in known biosynthetic pathways are more likely to affect virulence at the transcriptional and translational levels. For example, the *rpoC* and *miaA* genes the functions of which are discussed above.

Finally, since most of the selected genes are involved in metabolic steps absent from the human metabolism network, many metabolic genes could be targets for anti-infective therapies against *P. aeruginosa* infections. Nevertheless, it is still debatable whether prioritization in pharmacological targeting should be given to virulence-related metabolic genes that also affect growth or those that do not affect growth.

6.3 Extension of the main project

In a parallel investigation, we found differential expression of many known virulence factors and some metabolic genes through classical transcriptome analysis of highly versus lowly pathogenic *P. aeruginosa* strains. Combinatorial functional transcriptomics analysis at the pathway level was much more informative, revealing metabolic pathways containing differentially expressed genes between all 3 highly virulent versus all 3 lowly virulent strains

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and genes required for full virulence: (i) the 4-hydroxyl-phenylacetate degradation and succinate production, (ii) glutamine biosynthesis from glutamic acid, (iii) shikimate and chorismate biosynthesis from D-erythrose 4-phosphate, (iv) superpathway of branched-chain amino acid biosynthesis of valine, leucine and isoleucine, (v) 2,5- and 3,5-xylenol degradation to citramalate, and (vi) beta-oxidation of fatty acids. Thus, *P. aeruginosa* virulence can be analyzed at the transcriptome and functional level using common core metabolism modules that control and indicate the virulence of disparate *P. aeruginosa* strains.

Table 6. Phenotypes of virulence-related PA14 metabolic mutants. The table summarizes the results regarding the gene expression of virulence factors (exoT, exsC, pilA, pa1L) as well as the swarming and twitching motility phenotypes, in the virulence-related PA14 metabolic mutants. The asterisks indicate the significance compared to the wild-type strain: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to WT by Mann-Whitney *U* test.

#	Genes/Names	exoT	exsC	pilA	pa1L	Swarming motility	Twitching motility
1	PA14_17450 (*surE*)	*	*	*		***	**
2	PA14_64220 (*purD*)			*			
3	PA14_52050 (*purN*)	*		*		***	**
4	PA14_08780 (*rpoC*)	**	**	*	*	**	**
5	PA14_31700 (*pgsA*)						**
6	PA14_57780 (*hisD*)	*					
7	PA14_32690 (*gtdA*)			*		***	**
8	PA14_16070 (*hom*)	*				***	
9	PA14_05590 (*metF*)	*	*			***	
10	PA14_07170 (*epd*)	*	*			***	*
11	PA14_07600 (*folk*)	**				***	*
12	PA14_68580 (*pckA*)	*					*
13	PA14_65320 (*miaA*)	**	**	*	*		**

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CHAPTER 7

APPENDICES

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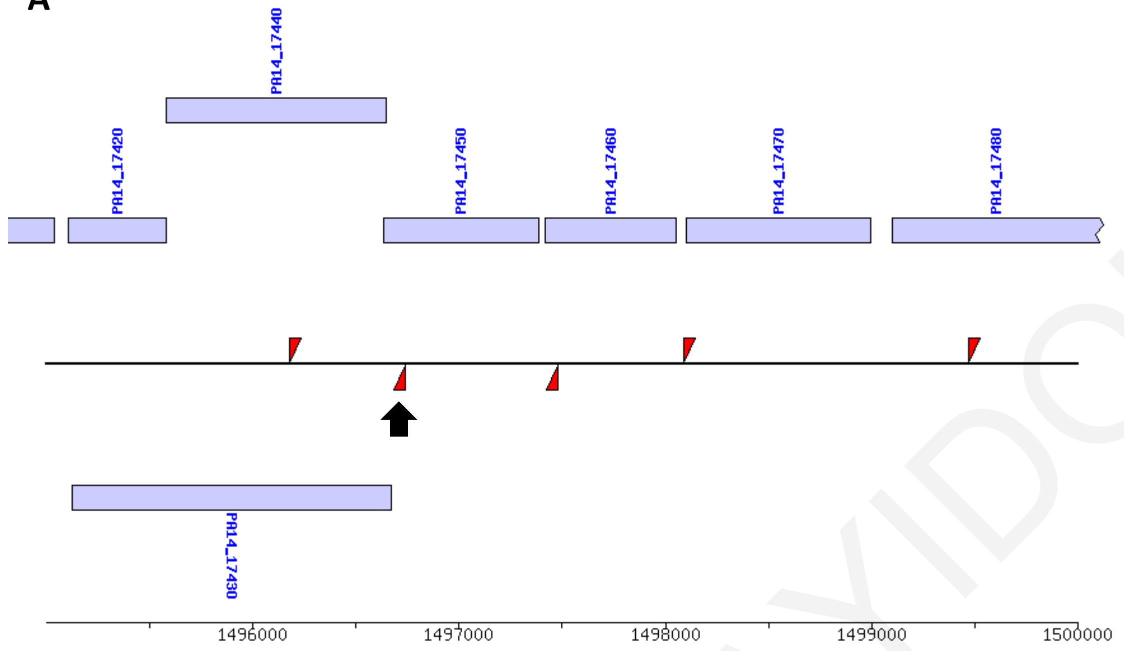

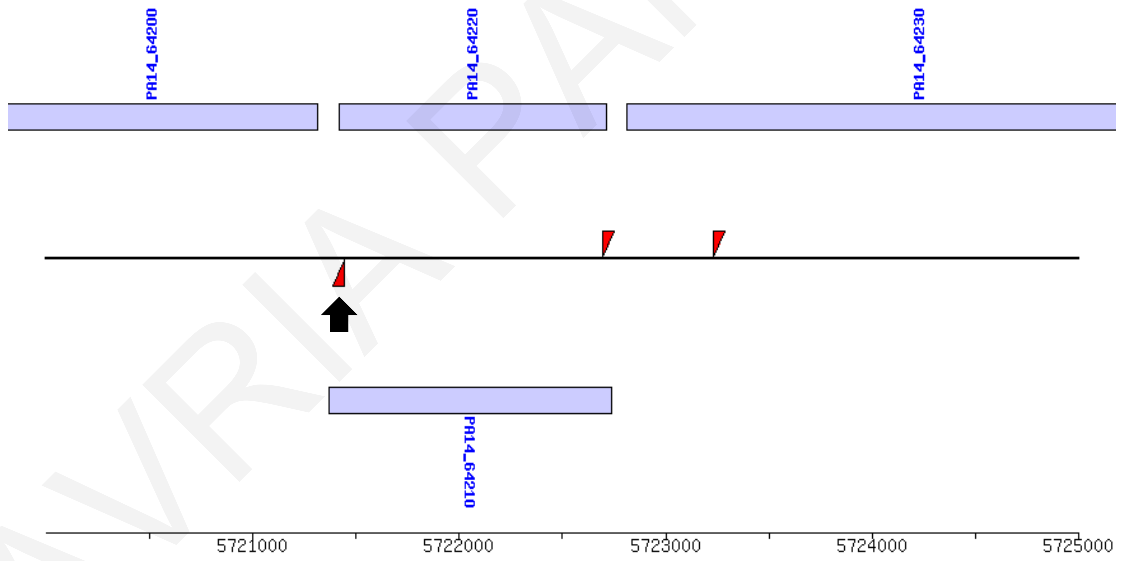

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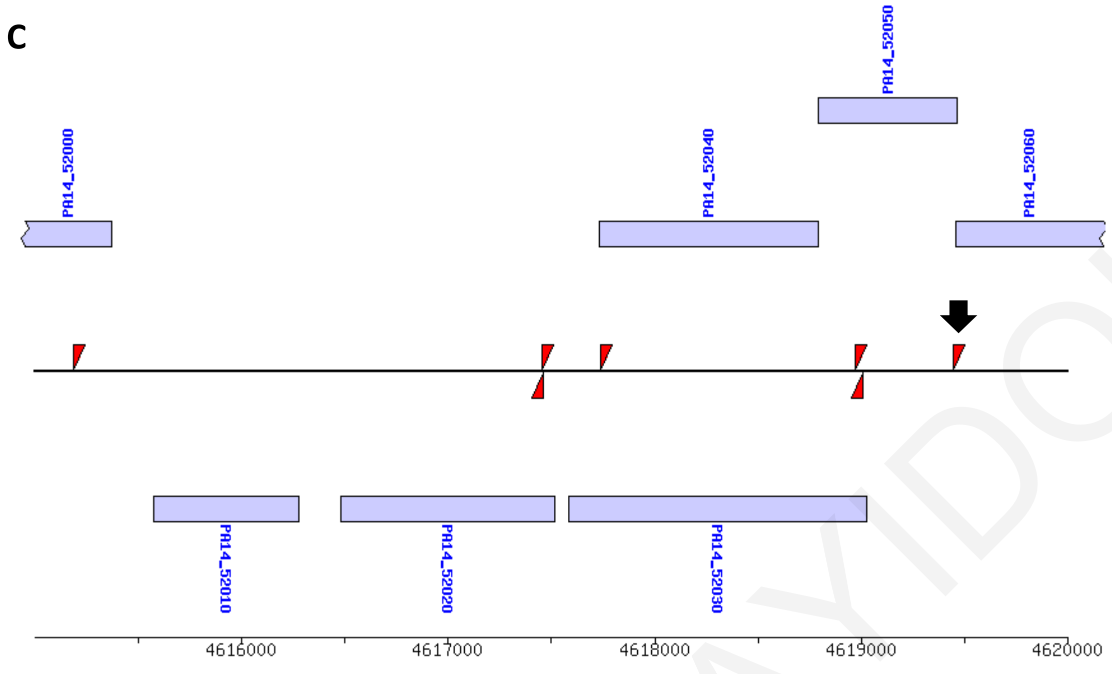

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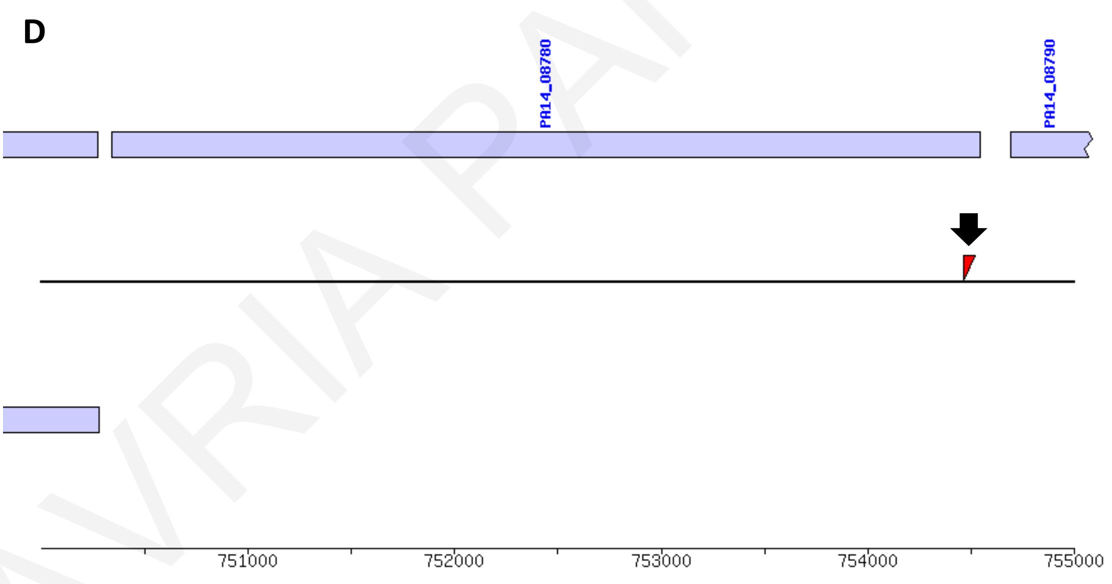

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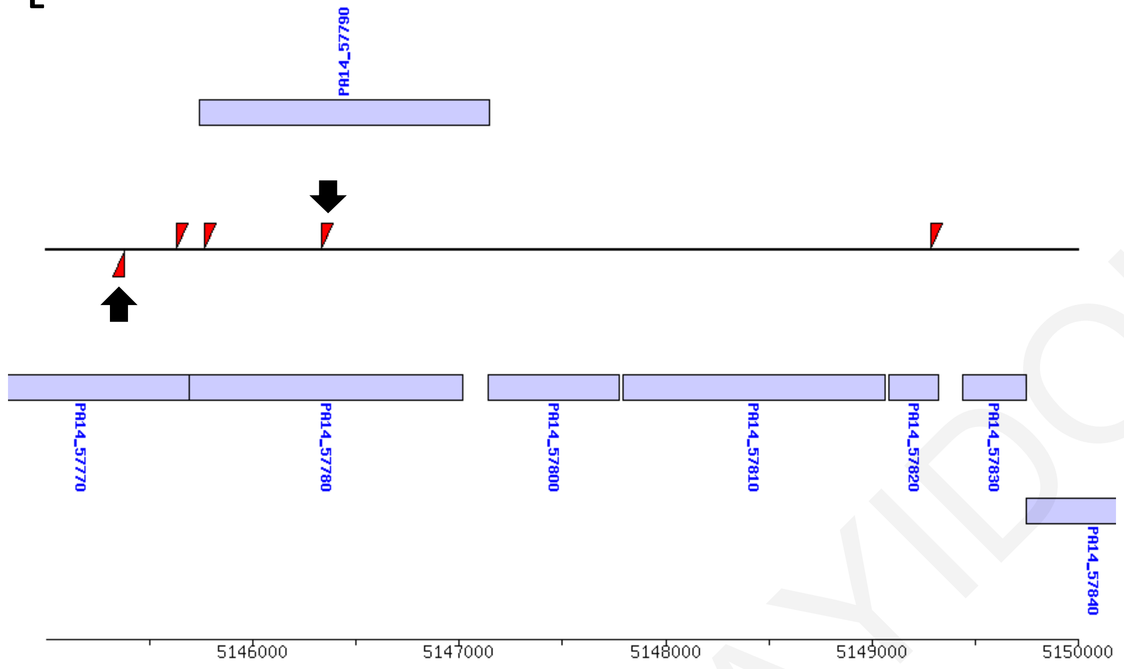

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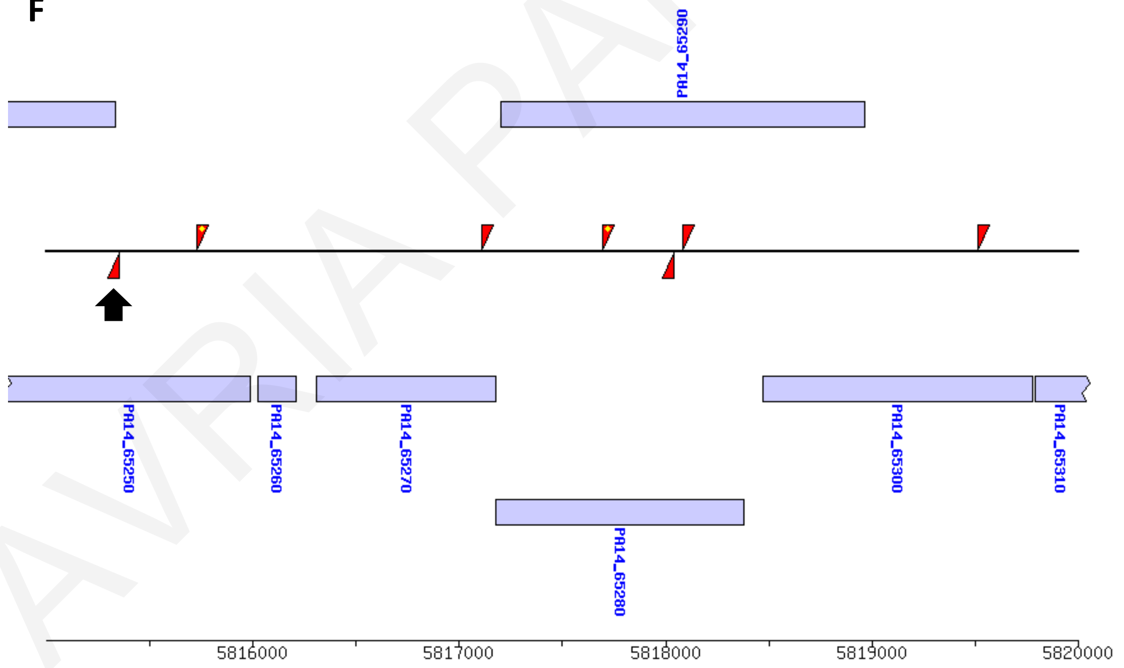

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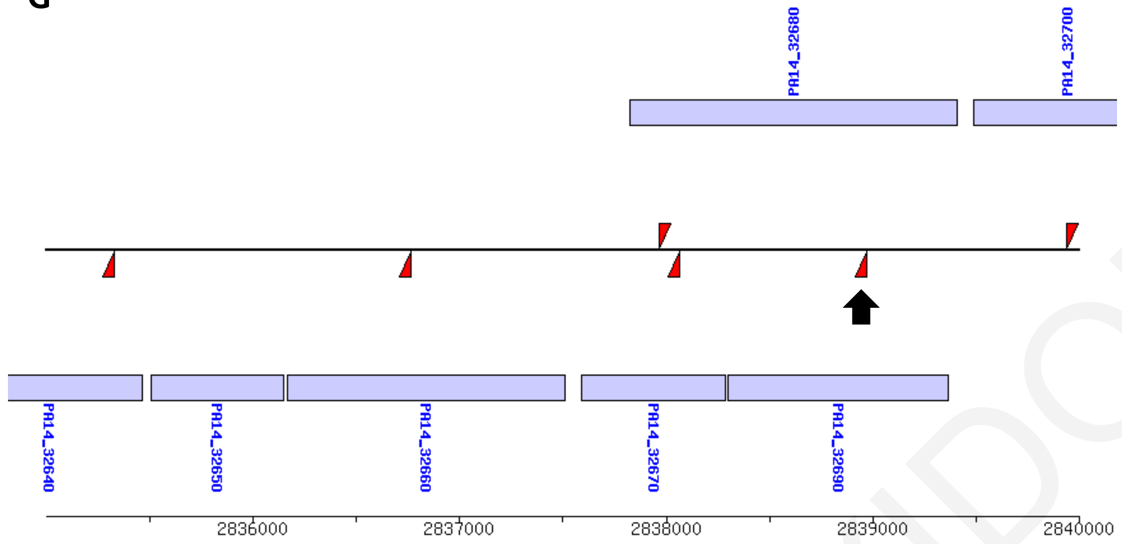

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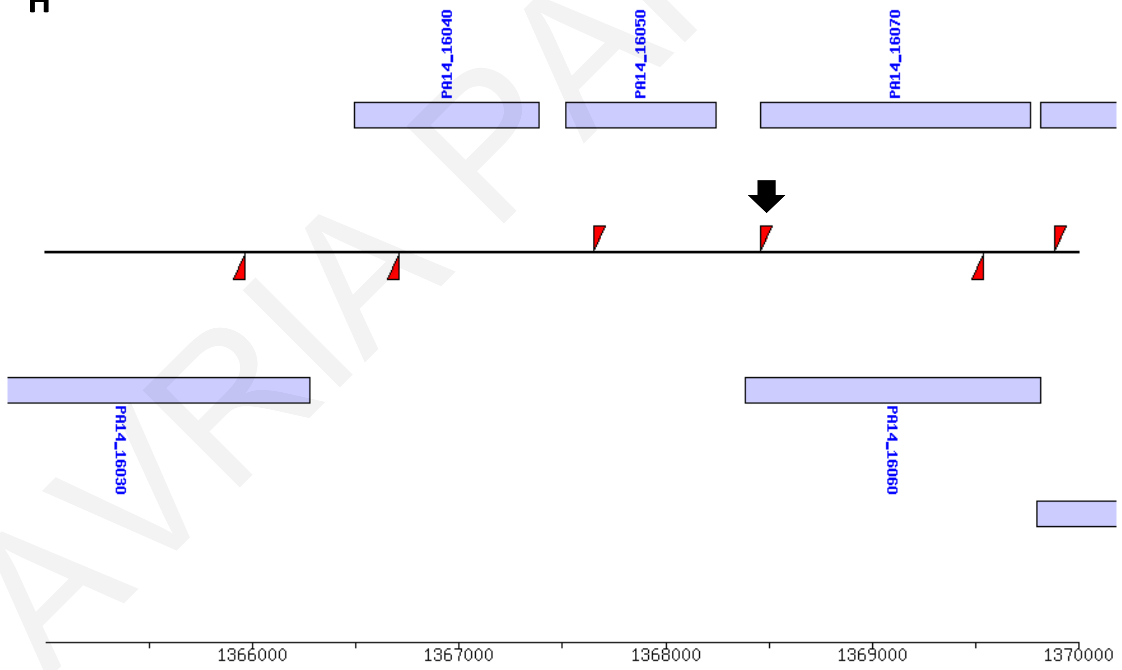

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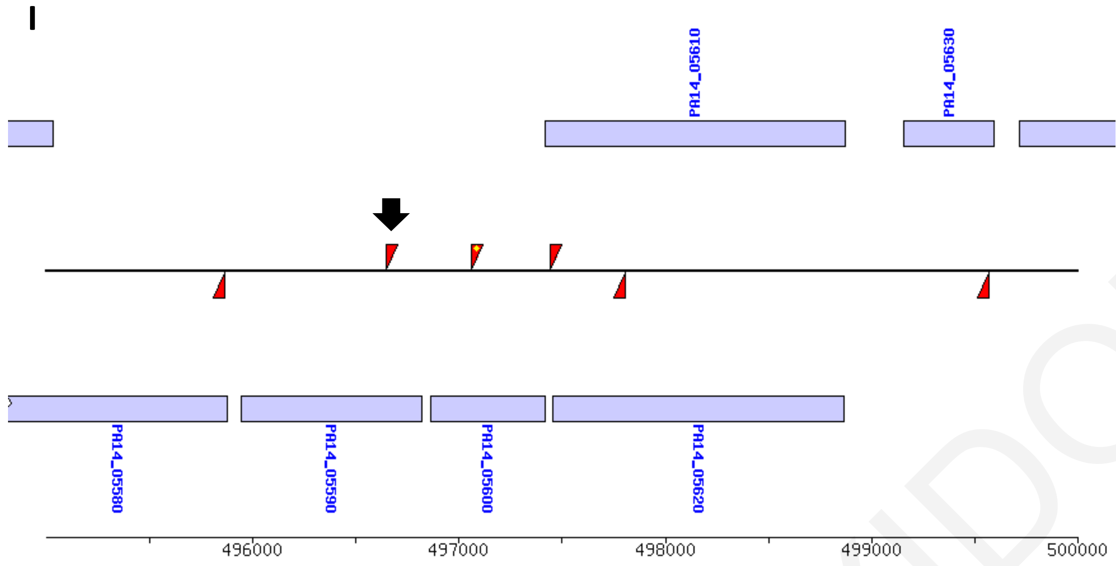

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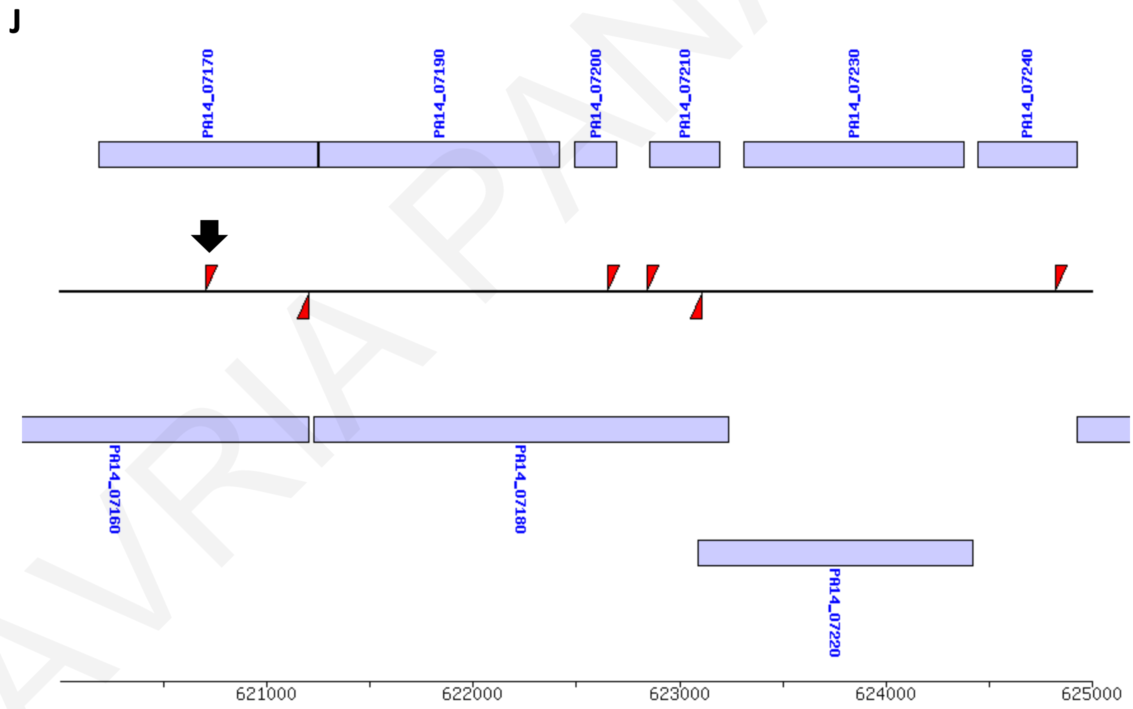

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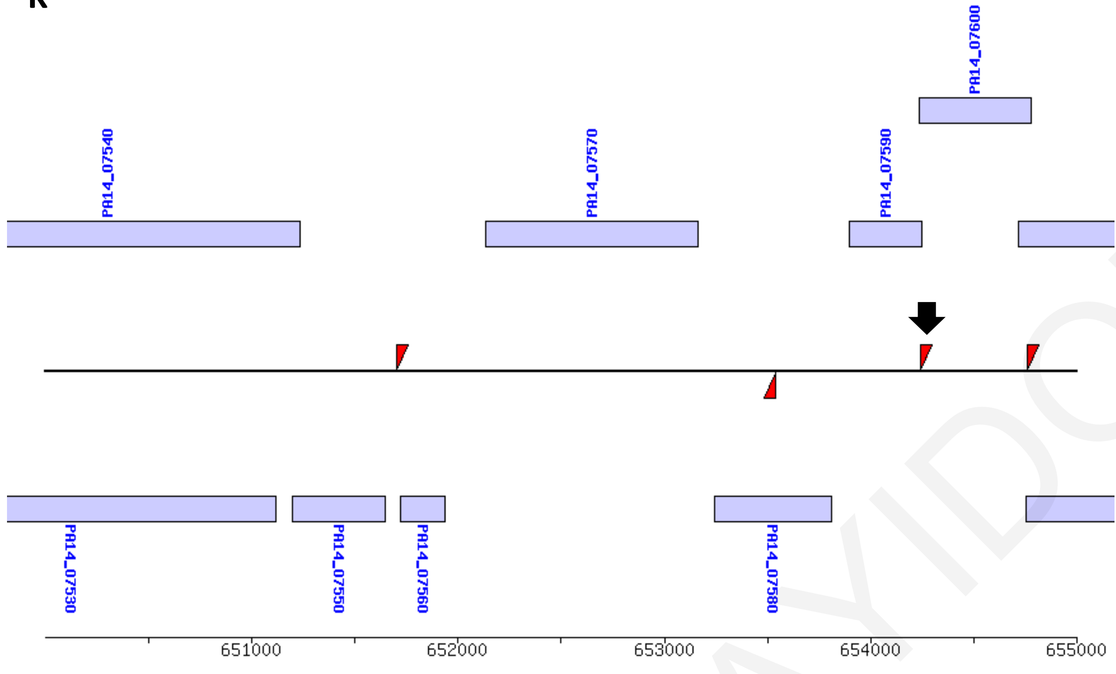

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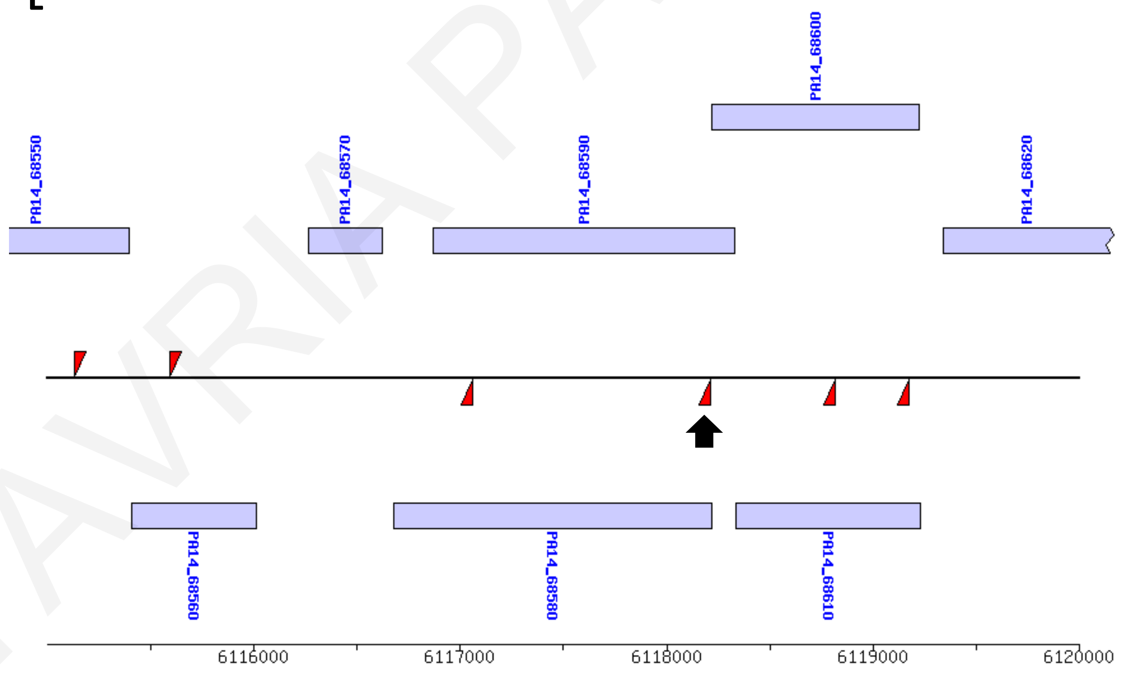

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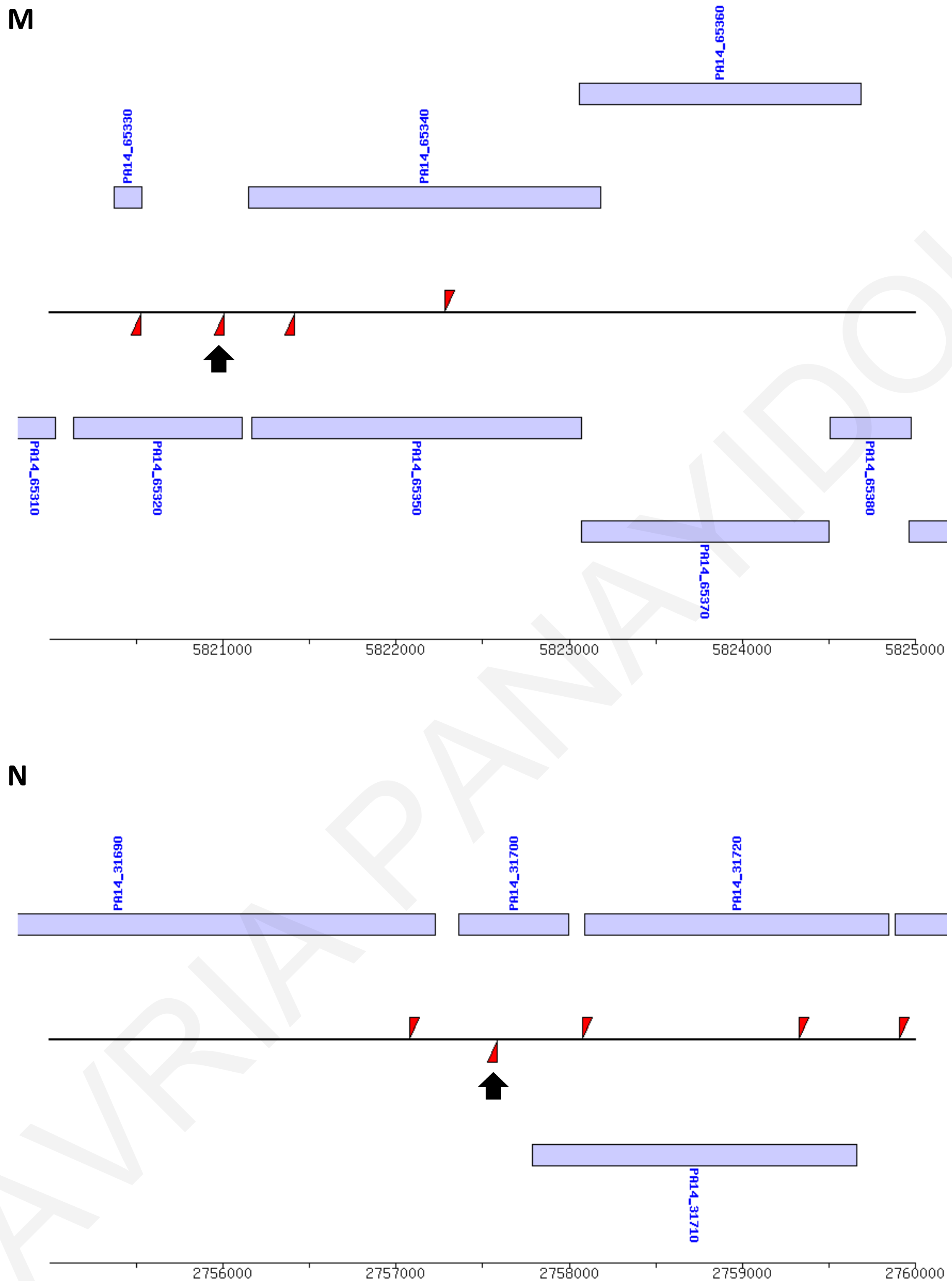

Figure 29. Transposon insertion sites of the 15 virulence-related PA14 metabolic genes. The line represents the PA14 genomic sequence. Genes (rectangles) and transposon insertions (triangles) pictured above the line are oriented 5' to 3' from left to right. Genes and insertions pictured below the line are oriented 5' to 3' from right to left. Transposon insertion sites of the virulence-related PA14 metabolic genes (listed in **Table 4**) are shown by black arrows (A-N).

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CHAPTER 8

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CHAPTER 9

PUBLICATIONS

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1. **Panayidou Stavria** *et al. Pseudomonas aeruginosa* core metabolism exerts a widespread growth-independent control on virulence. https://www.biorxiv.org/content/10.1101/850206v1

2. Christofi T., **Panayidou Stavria**, Dieronitou I., Michael C. & Apidianakis Y. Metabolic output defines *Escherichia coli* as a health-promoting microbe against intestinal *Pseudomonas aeruginosa*. Scientific Reports 9, Article number: 14463, 2019 October 8.

3. **Panayidou Stavria** & Apidianakis Y. Laboratory Models of Foodborne Infections, Chapter 25: *Pseudomonas aeruginosa*, Food Microbiology Series, CRC Press, 2017 March 21.

4. **Panayidou Stavria**, Ioannidou E & Apidianakis Y. Human pathogenic bacteria, fungi and viruses in Drosophila: disease modeling, lessons and shortcomings. Virulence, 2014 Jan 7; 5(2).

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6. **Panayidou Stavria** & Apidianakis Y. Regenerative Inflammation: Lessons from Drosophila Intestinal Epithelium in Health and Disease. Pathogens, 2013; 2(2):209-231.

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Metabolic output defines *Escherichia coli* as a health-promoting microbe against intestinal *Pseudomonas aeruginosa*

Theodoulakis Christofi, Stavria Panayidou, Irini Dieronitou, Christina Michael & Yiorgos Apidianakis

Gut microbiota acts as a barrier against intestinal pathogens, but species-specific protection of the host from infection remains relatively unexplored. Although lactobacilli and bifidobacteria produce beneficial lactic and short-chain fatty acids in the mammalian gut, the significance of intestinal *Escherichia coli* producing these acids is debatable. Taking a Koch's postulates approach in reverse, we define *Escherichia coli* as health-promoting for naturally colonizing the gut of healthy mice and protecting them against intestinal colonization and concomitant mortality by *Pseudomonas aeruginosa*. Reintroduction of faecal bacteria and *E. coli* in antibiotic-treated mice establishes a high titre of *E. coli* in the host intestine and increases defence against *P. aeruginosa* colonization and mortality. Strikingly, high sugar concentration favours *E. coli* fermentation to lactic and acetic acid and inhibits *P. aeruginosa* growth and virulence in aerobic cultures and in a model of aerobic metabolism in flies, while dietary vegetable fats - not carbohydrates or proteins - favour *E. coli* fermentation and protect the host in the anaerobic mouse gut. Thus *E. coli* metabolic output is an important indicator of resistance to infection. Our work may also suggest that the lack of antimicrobial bacterial metabolites in mammalian lungs and wounds allows *P. aeruginosa* to be a formidable microbe at these sites.

Escherichia coli and streptococci are the first bacteria to colonize the gastrointestinal tract of humans upon birth, paving the way for the establishment of species of the *Bifidobacterium*, *Bacteroides* and other genera[1]. *Bifidobacterium* and *Lactobacillus* strains are considered efficient fermenters in the human gut[2,3]. *E. coli* on the other hand thrives aerobically, but may also ferment carbon sources anaerobically to produce short-chain fatty acids (SCFAs) such as acetic acid and related metabolic products such as lactic acid[4,5]. While it is an effective colonizer of the healthy anaerobic mammalian gut, commensal *E. coli* also has a fitness advantage upon gut inflammation and concomitant host-derived nitrate production[5]. Interestingly, the probiotic *E. coli* strain Nissle 1917 (EcN) is particularly beneficial to ulcerative colitis patients in maintaining disease remission[6–8]. EcN induces host immune defence against pathogens[9,10], strengthens the intestinal barrier[11,12], and directly inhibits pathogenic *E. coli* strains[13,14]. Yet the beneficial role of *E. coli* has so far only been demonstrated for EcN and is not linked to lactic acid and SCFA production, while lactic acid bacteria, such as lactobacilli and bifidobacteria, are considered the main probiotic fermenters in the mammalian gut.

Antibiotics can greatly reduce microbiota diversity and promote dysbiosis early in life[15]. In children and adults, opportunistic pathogens can take advantage of the antibiotic effect on commensal bacteria to infect the gut[16]. One such pathogen is the gram-negative human opportunistic bacterium *Pseudomonas aeruginosa*, which is frequently found in hospital-acquired infections[17]. While not a common clinical problem in the gut, *P. aeruginosa* colonizes the gastrointestinal tract of many hospitalized patients and to a lesser extent of healthy individuals[18–21]. *P. aeruginosa* can nevertheless cause frequent and severe wound and lung infections in immunocompromised individuals and the ears and eyes of seemingly healthy people[22]. It is responsible for more than 50,000 infections per year in the U.S. alone, causing acute, chronic and relapsing infections due to a wide variety of virulence factors. Many of its virulence genes are controlled by quorum sensing (QS), a bacterial communication system

Department of Biological Sciences, University of Cyprus, Nicosia, Cyprus. Correspondence and requests for materials should be addressed to Y.A. (email: apidiana@ucy.ac.cy)

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that promotes synchronized microbial behaviours such as the production of the oxidative agent pyocyanin by *P. aeruginosa*[23].

Here we interrogate the contribution of *E. coli* in controlling *P. aeruginosa* intestinal colonization in a nutrient-dependent manner. We apply the Koch's postulates in reverse to prove a causal role of commensal *E. coli* in fending off *P. aeruginosa* infection. We found that: (**a**) *E. coli* is detectable through culture-independent methods (16S sequencing) in the faeces of untreated mice but not of antibiotic-treated mice, which become susceptible to infection; (**b**) A candidate health-promoting commensal *E. coli* strain was isolated through culture-dependent microbiological analysis and archived as a pure culture in the laboratory; (**c**) This mouse *E. coli* strain and other *E. coli* strains ameliorate *P. aeruginosa* infection when introduced into antibiotic-treated mice; (**d**) The administered health-promoting *E. coli* strains can be identified in high titres in the faeces of mice in which resistance to infection was improved. Moreover, assessing three extremes and a conventional diet in mice we find that, while sugars are fermented by various *E. coli* strains to lactic and acetic acid in culture and in flies aerobically, in the anaerobic mouse gut a vegetable-fat-based rather than a carbohydrate- or protein-based diet boosts lactic acid production and helps *E. coli* to inhibit *P. aeruginosa*. Our findings support the notion that unbalanced diets or the use of antibiotics may eliminate not only lactic acid bacteria but also commensal *E. coli*, imposing a gut environment conducive to *P. aeruginosa* infection due to the depletion of lactic acid and SCFAs.

Methods

Bacterial strains. *Pseudomonas aeruginosa* strain UCCBP 14 (PA14) and isogenic gene deletion mutants *Δmvfr*, *ΔphzS*, *ΔphzS* and *ΔrhlR*/*ΔlasR* were previously described[24,25]. *E. coli* MGH is a human isolate obtained from Prof. Elizabeth Hohmann at Mass General Hospital (Boston, USA). Mouse *E. coli* (*E. coli* CD1) was isolated from the faeces of CD1 mice for this study and validated through colony PCR and biochemical analysis i.e. being positive for indole production and growth on selective chromogenic Tryptone Bile X-glucuronide (TBX) agar plates. Laboratory *E. coli* BW25113 and KEIO collection strains, including *Δpgi*, *ΔadhE*, *ΔatpC*, *Δpta* and *ΔldhA*, were previously described[26]. Laboratory *E. coli* BW25113 and *Δtna*, *ΔsdiA*, *ΔluxS*, strains were previously described[27]. Enteropathogenic (EPEC) *E. coli* O127:H6 E2348/69 was obtained from Prof. Tassos Economou and was previously described[28].

Bacteria handling for in-culture experiments. *E. coli* and *P. aeruginosa* strains were grown at 37 °C overnight with shaking at 200 rpm in liquid LB from frozen LB-20% glycerol stocks. Cultures were then diluted to OD_{600nm} 0.01 in fresh sterile LB to establish mono- or co-cultures. Sucrose or glucose was added to a final concentration of 4% w/v during growth assessments. Bacterial supernatants were produced by overnight bacterial cultures filter-sterilized and mixed in 1:1 volume ratio with fresh LB broth. Selective plates contained 50 μg/ml rifampicin for *P. aeruginosa* and 60 μg/ml kanamycin for *E. coli* Keio collection or TBX agar for wild-type *E. coli*.

Fly survival. For aerobic growth, strains were grown at 37 °C overnight with shaking at 200 rpm in liquid LB from frozen LB-20% glycerol stocks and then diluted to OD_{600nm} 0.01 in fresh sterile LB grown over day to OD_{600nm} 3. For anaerobic growth, strains were grown at 37 °C for 72 hours without shaking in liquid BHI from frozen BHI-20% glycerol stocks to OD_{600nm} 1–2. Cultures were then pelleted and diluted to a final OD_{600nm} 0.15 per strain in a 4% sugar (sucrose or glucose), 10% sterile LB infection medium. Wild-type Oregon R *Drosophila melanogaster* female flies 3–5 days old were starved for 6 hours prior to infection. 5 ml infection medium was added on a cotton ball at the bottom of a fly vial. Each vial contained 10 to 15 flies and observed twice a day for fly survival[29].

Fly colonization. Germ-free flies were generated through dechorionation of collected eggs in 50% bleach. Adult Oregon R 3–5-day-old female flies were infected for 24 hours with a single bacterial culture or a mix of cultures grown as mentioned above, pelleted and diluted to a final OD_{600nm} 0.02 per strain in a 4% sugar (sucrose or glucose) medium. Flies were then transferred to modified falcon tubes and maintained there with 200 μl 2% or 4% of sucrose or glucose as previously described[24]. At day 2 and day 5 flies were homogenized using the Qiagen Tissuelyser LT for 5 minutes at 50 Hz. Bacteria CFUs were enumerated on selective plates after overnight incubation at 37 °C.

KEIO *E. coli* gene deletion library screen. The Keio *E. coli* collection of gene knockouts was acquired from the Japanese National Institute of Genetics and contains 3884 *E. coli* mutants with unique gene deletions. Strains were grown overnight in sterile 96-well clear flat bottom plates containing 200 μl of sterile LB broth at 37 °C and 200 rpm shaking. *P. aeruginosa* was grown in glass tubes at standard overnight conditions. Over day co-cultures were incubated at 37 °C and 200 rpm in 96-well plates starting with 1:100 dilutions of *P. aeruginosa* and *E. coli* mutant overnight cultures in 200 μl LB broth supplemented with 4% glucose. At 24 hours pyocyanin production was observed visually using as positive controls PA14 monocultures and co-cultures of PA14 with *E. coli* mutants lacking inhibitory properties (e.g. *Δpgi*). Bacterial growth was measured at OD_{600nm} on a plate reader. Bacterial co-cultures typically exhibit half the optical density of PA14 monocultures. Thus co-cultures with optical density equal to or higher than PA14 monocultures indicated antagonistic interactions.

Animal diets. *Drosophila melanogaster* Oregon R flies were reared in a cornmeal, yeast and sugar diet at 25 °C in a 12-hour day and night cycle. CD1 mice were reared 5–6 individuals per cage at 24 °C in a 12-hour day and night cycle. Standard chow diet was obtained from Mucedola s.r.l Italy (#4RF25 a complete balanced diet containing mainly starch 35.18%, sucrose 5.66%, crude protein 22%, crude oil 3.5%). Specialized diets based on either vegetable fats, carbohydrates or protein were manufactured by Mucedola s.r.l (#PF4550, PF4551 and PF4552) per Table 1 below[30].

	Carbohydrate	Fat	Protein
Corn starch	58.11	0.00	0.00
Powdered sugar	29.06	0.00	0.00
Casein	0.00	0.00	87.17
dl-Methionine	0.11	0.20	0.11
Vegetable shortening*	0.00	75.12	0.00
AIN-76A vitamin mix**	0.77	1.49	0.77
AIN-76A mineral mix**	3.07	5.95	3.07
Choline chloride	0.18	0.34	0.18
Cellulose (Alphacel)	8.72	16.91	8.72
Energy density, kcal/g	3.53	6.85	3.53

Table 1. Composition of macronutrient diets (% by weight). *Crisco brand, a blend of soybean oil, fully hydrogenated palm oil, and partially hydrogenated palm and soybean oils. Contains 50% polyunsaturated fat, 20.8% monounsaturated fat, 0% trans fat and 25% saturated fat per weight. **Vitamin (A and D3) and mineral (Fe, Mn, Zn, Cu, I, Se) mixes contain 97% and 12% sucrose, respectively.

Ethics statement. Animal protocols were approved by the Cyprus Veterinary Service inspectors under the license number CY/EXP/PR.L6/2018 for the Laboratory of Prof. Apidianakis at the University of Cyprus. The veterinary services act under the auspices of the Ministry of Agriculture in Cyprus and the project number is CY.EXP101. These national services abide by the National Law for Animal Welfare of 1994 and 2013 and the Law for Experiments with Animals of 2013 and 2017. All experiments were performed in accordance with these guidelines and regulations.

Mouse colonization assay. Female CD1 mice 7–8 weeks old were treated with an antibiotic cocktail of 0.1 mg/ml Rifampicin, 0.3 mg/ml Ampicillin and 2 mg/ml Streptomycin for 6 days to reduce endogenous gut bacteria. Subsequently, PA14 was provided daily for 7 days in the drinking water prepared from an over-day culture of OD_{600nm} 3, centrifuged at 4610 RCF for 5 minutes to collect bacteria and diluted 1:10 to obtain ~3×10^8 bacteria/ml. Following infection (Day 0 of PA14 colonization) *E. coli* was provided for 1 day at the same concentration and CFUs for both bacteria were measured every other day from homogenized and plated mouse faeces.

16S Metagenomic. Mouse faecal samples were collected in Eppendorf tubes, weighed, snap frozen and stored at −80 °C. Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). 16S Sequencing was performed using the Illumina metagenomics analyser. Kraken software was used to assign taxonomic sequence classification.

Mouse survival assay. Female CD1 mice 7–8 weeks old were given an antibiotic cocktail of 0.1 mg/ml Rifampicin, 0.3 mg/ml Ampicillin and 2 mg/ml Streptomycin in their drinking water for 6 days to reduce endogenous gut bacteria. Subsequently, *E. coli* strains were provided in drinking water for 24 hours prepared from an over-day culture of OD_{600nm} 3 and/or anaerobic faecal culture grown to its maximum for 2 days, centrifuged at 4610 RCF for 5 minutes to collect bacteria and diluted 1:10 to obtain ~3×10^8 bacteria/ml. The next day *P. aeruginosa* (strain PA14) was provided daily for 7 days in the drinking water as for *E. coli*. Then mice were injected intraperitoneally with 150 mg/kg of body weight with cyclophosphamide (CP) and 3 days later with another dose of 100 mg/kg as previously described[31]. Survival was observed twice a day until all mice die or for up to 1 week.

Acid and sugar measurements. Lactic and acetic acid concentrations in culture supernatants and homogenized mouse faeces (produced via bead homogenization in water) were determined enzymatically using R-Biopharm kits No. 11112821035 and No. 10148261035 respectively, according to manufacturer's instructions. Sugar concentrations in homogenized mouse faeces were determined using the Megazyme Sucrose/D-Fructose/D-Glucose Assay Kit (K-SUFRG) according to manufacturer's instructions. Absorbance was measured using the NanoDrop 2000c Spectrophotometer.

Pyocyanin measurement. Overnight PA14 cultures were diluted to OD_{600nm} 1, then 0.25 ml was used to inoculate 25 ml of LB. Cultures were grown at 37 °C, 200 rpm in 250 ml flasks. Supernatants were collected after centrifugation at 4800 RCF for 10 minutes. 4.5 ml of chloroform was added to 7.5 ml of supernatant and vortexed. Samples were then centrifuged at 4800 RCF for 10 minutes. 3 ml of the resulting blue layer at the bottom was transferred to a new tube. 1.5 ml of 0.2 M HCl was added to each tube and vortexed 2 times for 10 seconds. Samples were centrifuged for 3 minutes at 4800 RCF and 1 ml of the pink layer was transferred to cuvettes. Pyocyanin concentration (μg/ml) was calculated by multiplying the spectrophotometric measurements taken at OD_{520nm} by 17.072, then multiplying them again by 1.5 due to the chloroform dilution.

Computational analysis. Pairwise comparisons of bacterial CFUs and other pairwise comparisons were evaluated using the two-sided Student's t-test for samples of ≥10 and Mann–Whitney U-test or one-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison test for samples <10. Survival curves of mice and flies were analysed with the Kaplan-Meier method and the log-rank test. All experiments were repeated at least twice with qualitatively similar results. Gene enrichment analysis was performed using the David's functional annotation tool. Correlation coefficient (*R*) significance analyses of mouse faecal acid concentration vs.

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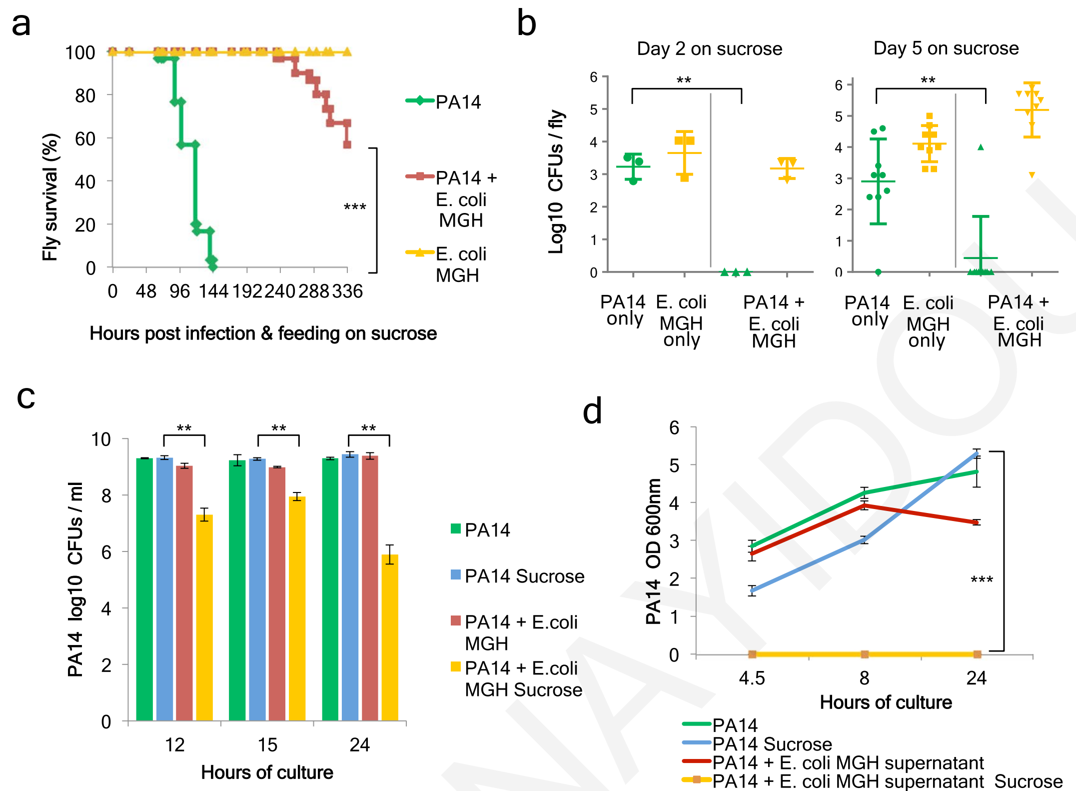

Figure 1. *E. coli* MGH inhibits *P. aeruginosa* growth and virulence in the *Drosophila* gut and in culture in the presence of sucrose. (**a**) Survival of *Drosophila melanogaster* Oregon R flies infected with PA14, *E. coli* strain MGH or co-infected with *E. coli* MGH and *P. aeruginosa* PA14 [n = 30]. (**b**) Colonization levels measured in colony forming units (CFUs) at Day 2 [n = 3] and Day 5 [n = 9] post-PA14-infection only, *E. coli* MGH only, and upon co-infection (triangles for PA14, inverted triangles for MGH). (**c**) CFUs of PA14 growth in the presence or absence of 4% sucrose and *E. coli* MGH in LB cultures [n = 3]. (**d**) Optical density measurements at 600 nm of PA14 growth in half fresh LB, half liquid supernatant of *E. coli* LB cultures +/− 4% sucrose [n = 9]. $**p < 0.005$, $***p < 0.0005$. Error bars represent standard deviation of the mean.

LT50 was done using Pearson correlation and an n = 6 (the average of six dietary conditions sampling 6 mice for each). The Acetic + Lactic acid Index for each of the 6 dietary conditions was computed by dividing each acid concentration of each dietary condition with the average concentration of that acid in all conditions and adding the normalized values of the two acids. For sucrose assimilation prediction we used BLASTN 2.8.1+ per Zhang *et al.* 2000[32] and found (a) an *E. coli W* sucrose hydrolase (98% identity), (b) a sucrose permease (98% identity), (c) a sucrose-specific IIBC component (100% identity) and (d) a sucrose-6-phosphate hydrolase (100% identity) present in *E. coli* O127:H6 str. E2348/69 (taxid:574521), but not in the genomes of *E. coli* BW25113 (taxid:679895) and *E. coli* DH5[alpha] (taxid:668369).

Results

Escherichia coli-secreted factors antagonize _Pseudomonas aeruginosa_ growth in the presence of sugars. Screening for bacterial strains that may alleviate *P. aeruginosa* infection in *Drosophila*, we compared and combined the highly virulent *P. aeruginosa* strain, PA14, which kills all orally infected flies within 6 days[29], with various *E. coli* strains (MGH, EPEC, BW25113 and DH5a), none of which was by itself significantly lethal to flies. The fly lethal time 50% (LT50%) extended beyond the 15 days for all *E. coli* strains, as exemplified with *E. coli* MGH and BW25113 shown in Fig. 1a and Suppl. Fig. 1a. Strikingly, *P. aeruginosa*-mediated fly lethality, fly colonization and *P. aeruginosa* growth in culture was dramatically inhibited by the human *E. coli* isolate MGH (Fig. 1a–d) and the laboratory *E. coli* strain BW25113 (Suppl. Fig. 1a–d) in the presence of 4% sucrose or 4% glucose, respectively. Noticeably, sucrose can be used by the *E. coli* strains MGH and EPEC to inhibit *P. aeruginosa* lethality and growth (fly LT50% > 10 days; Fig. 1a–d), because EPEC, for example, has 4 sucrose uptake and metabolism enzymes, namely, an *E. coli* W sucrose hydrolase, a sucrose permease, a sucrose-specific IIBC component and a sucrose-6-phosphate hydrolase. In contrast, the *E. coli* strains BW25113 and DH5a do not have these genes and were unable to utilize sucrose to inhibit *P. aeruginosa* in our experiments (fly LT50% < 7 days). As expected, when 4% glucose instead of sucrose was used in the infection mix, *E. coli* BW25113 gained the capacity to inhibit *P. aeruginosa* lethality, fly colonization and in culture growth (Suppl. Fig. 1a–d)[33].

Of note, *E. coli* antagonizes *P. aeruginosa* not only in fly survival but also in inhibiting its colonization. We assessed the bacterial loads of each bacterial strain independently in the fly and upon co-infection. Colony forming unit (CFU) measurements in selective media revealed that at 2 days and 5 days after infection with either

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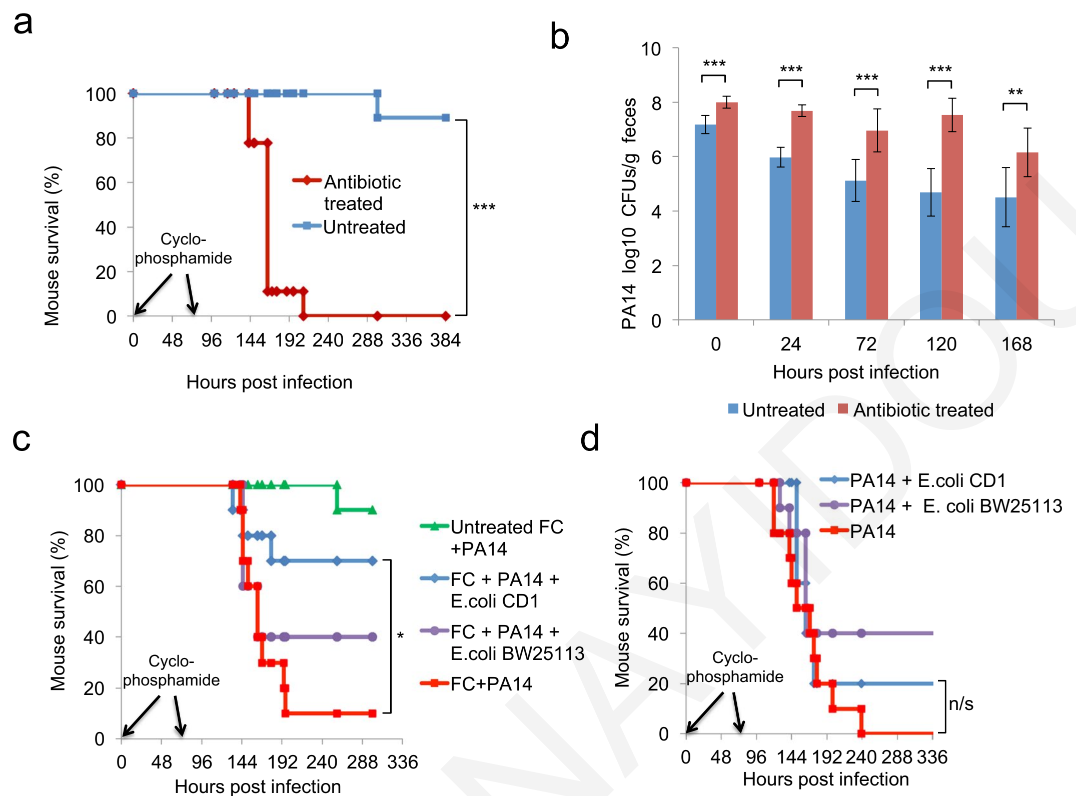
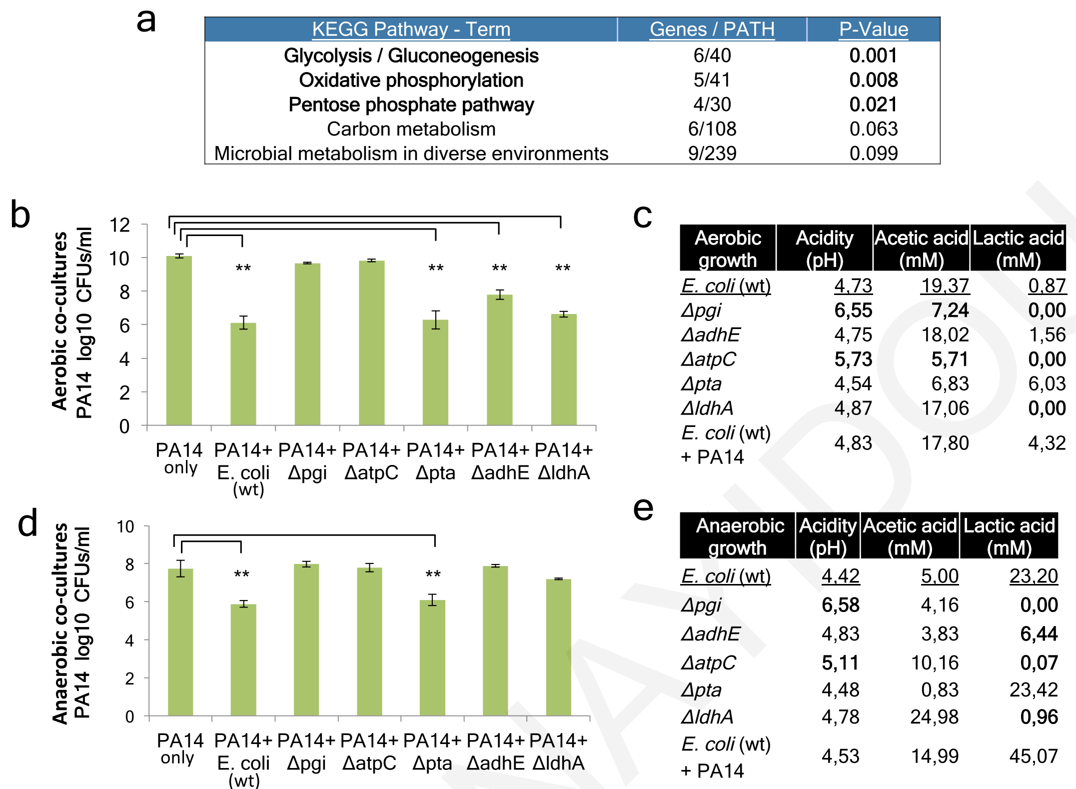


Figure 2. Commensal bacteria and *E. coli* protect antibiotic- and cyclophosphamide-treated mice from *P. aeruginosa*-induced lethality and colonization. (**a**) Survival to *P. aeruginosa* infection of immunocompromised mice pre-treated with antibiotics or untreated [n = 9]. (**b**) PA14 CFUs in faeces of immunocompromised mice pre-treated with antibiotics or untreated [n = 9]. (**c**) Survival to PA14 infection of immunocompromised mice feeding on a faecal culture (FC) fortified with *E. coli* BW25113 or mouse isolate *E. coli* CD1. Controls include antibiotic-treated and PA14-infected immunocompromised mice without *E. coli*, and mice without *E. coli* or antibiotic treatment [n = 10]. (**d**) Survival of antibiotic-treated PA14-infected immunocompromised mice feeding on the commensal mouse *E. coli* CD1 or the *E. coli* BW25113. Control immunocompromised mice were antibiotics-treated and infected with PA14, but no *E. coli* [n = 10]. n/s = $p > 0.05$, *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. Error bars represent standard deviation of the mean.

P. aeruginosa or *E. coli* MGH flies harboured roughly 10^3 bacteria or more per fly (Fig. 1b). Upon co-infection with *E. coli* MGH, *P. aeruginosa* was almost eradicated, while *E. coli* MGH remained stable (Fig. 1b). Similarly, co-infection with the *E. coli* BW25113 strain and *P. aeruginosa* (PA14) resulted in lower PA14 CFUs and tentatively lower BW25113 CFUs at day 5 (Suppl. Fig. 1b), suggesting that mutual inhibition at the level of colonization is possible.

To assess if the inhibition between *P. aeruginosa* and *E. coli* is direct, we assessed bacterial growth in aerobic LB cultures. Interestingly, *E. coli* MGH did not inhibit *P. aeruginosa* growth in plain liquid LB (Fig. 1c). To assess if sucrose added in the fly infection media as a standard carbon source for the flies would make a difference in bacterial interactions in culture, we supplemented the LB media with 4% sucrose. Strikingly, in the presence of sucrose, *P. aeruginosa* CFUs were reduced by >1,000 fold when co-cultured with *E. coli* MGH, but no inhibition was noticed in the absence of sucrose (Fig. 1c). The monosaccharides glucose and fructose enable also the *E. coli* strain BW25113 to inhibit *P. aeruginosa* (Suppl. Fig. 1c). To assess whether secreted factors are responsible for *P. aeruginosa* growth inhibition we grew *P. aeruginosa* in a mix of 50% fresh LB and 50% filtered LB supernatant from an overnight *E. coli* culture that was supplemented or not with 4% sugar. The mix containing supernatant of *E. coli* MGH grown in sucrose and that of *E. coli* BW25113 grown in glucose was able to completely inhibit the growth of *P. aeruginosa* for at least 24 hours (Fig. 1d, Suppl. Fig. 1d).

E. coli inhibits _P. aeruginosa_ intestinal colonization and lethality during mouse gut-derived sepsis. To model the antibiotic-induced dysbiosis of mammals we used a mouse assay of intestinal infection. We administered a regime of three broad-spectrum antibiotics in mice and assessed their gut microbiota at the genus level through 16S sequencing analysis. In the absence of antibiotics, the microbiota consisted primarily of *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, including *E. coli* (Suppl. Fig. 2a). Using colony PCR sequencing we verified the presence of an endogenous *E. coli* strain (naming the respective cultured strain CD1) and further identified 7 easy-to-culture and potentially beneficial strains belonging to the *Lactobaccillus*, *Bifidobacterium* and *Bacteroides* genera in the faeces of mice (Suppl. Fig. 2b). Antibiotic treatment induced dysbiosis, which is



KEGG Pathway - Term	Genes / PATH	P-Value
Glycolysis / Gluconeogenesis	6/40	**0.001**
Oxidative phosphorylation	5/41	**0.008**
Pentose phosphate pathway	4/30	**0.021**
Carbon metabolism	6/108	0.063
Microbial metabolism in diverse environments	9/239	0.099

Aerobic growth	Acidity (pH)	Acetic acid (mM)	Lactic acid (mM)
E. coli (wt)	4,73	19,37	0,87
Δpgi	**6,55**	**7,24**	**0,00**
ΔadhE	4,75	18,02	1,56
ΔatpC	**5,73**	**5,71**	**0,00**
Δpta	4,54	6,83	6,03
ΔldhA	4,87	17,06	**0,00**
E. coli (wt) + PA14	4,83	17,80	4,32

Anaerobic growth	Acidity (pH)	Acetic acid (mM)	Lactic acid (mM)
E. coli (wt)	4,42	5,00	23,20
Δpgi	**6,58**	4,16	**0,00**
ΔadhE	4,83	3,83	**6,44**
ΔatpC	**5,11**	10,16	**0,07**
Δpta	4,48	0,83	23,42
ΔldhA	4,78	24,98	**0,96**
E. coli (wt) + PA14	4,53	14,99	45,07

Figure 3. *E. coli* mutants deficient in inhibiting *P. aeruginosa* and their acetic and lactic acid production profiles. (**a**) Enrichment analysis of *E. coli* genes identified as necessary for PA14 inhibition using EASE Score, a modified Fisher Exact P-Value. (**b**,**d**) PA14 CFUs in co-cultures with *E. coli* BW25113 (WT) and isogenic gene mutants at 5 hours of aerobic growth (**b**) or 24 hours of anaerobic growth (**d**) [n = 6]. (**c**,**e**) Liquid culture media pH and acid concentration (mM) at 5 hours of aerobic growth (c) or 24 hours of anaerobic growth (**e**) [n = 6]. Bold values indicate deviation from the wild type of mutant *E. coli* strains. **p < 0.005. Error bars represent standard deviation of the mean.

exemplified by the eradication of *E. coli*, the reduction of all the prevalent phyla below the detection level (Suppl. Fig. 2c), and the eradication of all 8 cultured bacterial strains except for *Bifidobacterium sp.2*, which was reduced from 8.4 $\log_{10}$ to 7.2 $\log_{10}$ CFUs per gram of mouse faeces (Suppl. Fig. 2d).

Antibiotic-treated mice subjected to immunosuppression via cyclophosphamide injections and infected with *P. aeruginosa* exhibit systemic spread of bacteria (Suppl. Fig. 3a–c) and die from sepsis as previously described[31]. Notably, all immunosuppressed dysbiotic mice died within 9 days of oral infection with *P. aeruginosa*, while 90% of the *P. aeruginosa*-infected immunocompromised mice that are not treated with antibiotics survived (Fig. 2a). Accordingly, *P. aeruginosa* load in the stools of infected mice bearing the healthy microbiota were significantly less at all time points than in mice treated with antibiotics, suggesting that commensal microbes inhibit colonisation by *P. aeruginosa* (Fig. 2b). To partly re-establish the mouse microbiome, we administered a faecal culture supplement (FC) prepared from a pelleted anaerobic stool culture. FC contained the endogenous *Bacteroides*, *Bifidobacteria* and *Lactobacillus* species and to a lesser extent the endogenous *E. coli*. The addition of FC in the drinking water had little to no effect by itself in protecting mice against lethality. However, FC fortified with the endogenous *E. coli* strain (*E. coli* CD1) rescued 70% of mice (Fig. 2c). On the other hand, the *E. coli* CD1 in the absence of FC did not protect mice against *P. aeruginosa* infection (Fig. 2d), suggesting a synergism between the endogenous *E. coli* CD1 and other members of the microbiota as a result of adaptation or co-evolution. Unlike the *E. coli* CD1 strain, the laboratory *E. coli* strain BW25113 showed only a trend in improving mouse survival due to *P. aeruginosa* infection, and this effect was not modifiable by FC (Fig. 2c,d). Despite the marginal effect on survival, *E. coli* BW25113 can stably colonize the mouse gut (Suppl. Fig. 3d) and reduces the *P. aeruginosa* burden significantly in the mouse gut within a week post-infection (Suppl. Fig. 3e).

Aerobic or anaerobic fermentation of glucose to lactic and acetic acid by *E. coli* is necessary for inhibiting *P. aeruginosa* growth. *E. coli* QS signalling and the production of the metabolite indole have been reported to inhibit *P. aeruginosa* growth[27,34]. To reveal *E. coli* factors that inhibit *P. aeruginosa* in our glucose-supplemented media, we assessed *E. coli* QS mutants and indole production genes previously implicated in bacterial competition[27]. We found that the *E. coli* QS genes *luxS* and *sdiA* are not necessary for *P. aeruginosa* inhibition in an LB culture supplemented with 4% glucose (Suppl. Fig. 4a). In addition, a deletion of the indole production enzyme tryptophanase (*tna*) essentially eliminated indole production (Suppl. Fig. 4b), but not the

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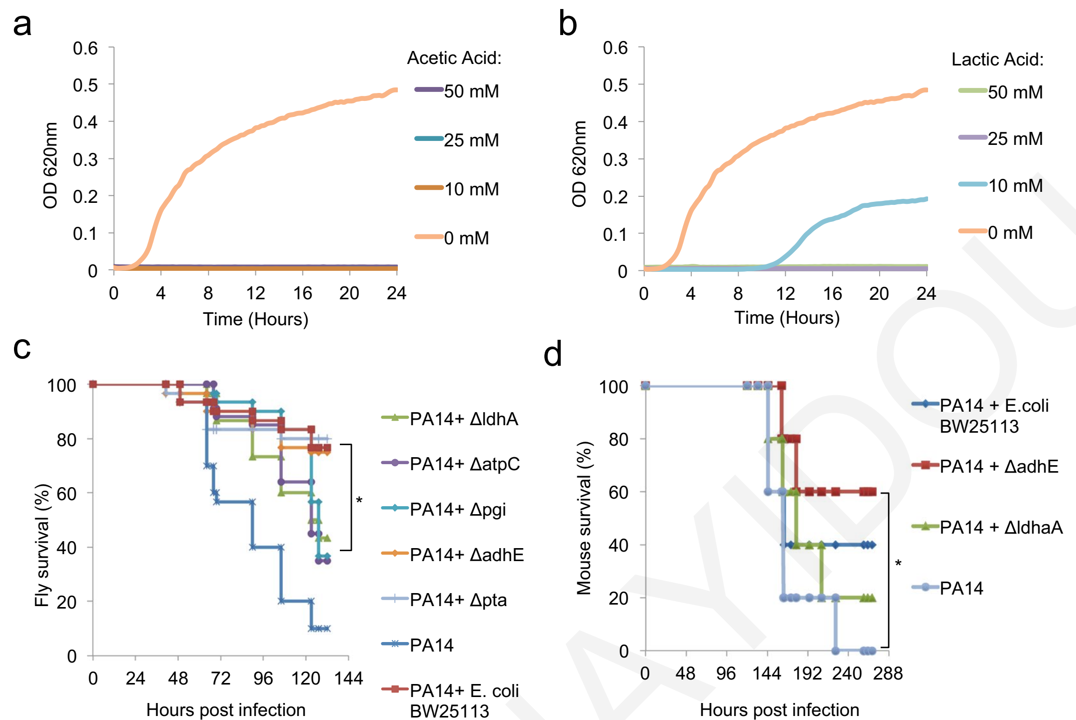

Figure 4. Lactic and acetic acid capacity to inhibit *P. aeruginosa* growth in culture media and virulence in flies and mice. (**a**,**b**) PA14 growth in LB broth supplemented with 0, 10, 25 and 50 mM of acetic acid (**a**) or lactic acid (**b**) at pH 5 [n = 4]. (**c**) *Drosophila* survival curves upon co-infection with PA14 and wild-type (BW25113) or isogenic mutant *E. coli* strains [n = 30]. (**d**) Survival of PA14-infected immunocompromised mice complemented with a faecal culture (FC) and wild-type *E. coli* BW25113 or *E. coli* mutants, *ΔldhA* or *ΔadhE*, or no *E. coli* [n = 10]. $^*p < 0.05$.

ability of *E. coli* BW25113 to inhibit *P. aeruginosa* (Suppl. Fig. 4c). Therefore we performed an unbiased screen of the KEIO collection of 3985 isogenic K-12 BW25113 gene mutants, identifying 45 genes that are necessary for the inhibition of *P. aeruginosa* in LB broth supplemented with 4% glucose. Gene enrichment analysis pinpointed glycolysis and the downstream pathways of oxidative phosphorylation and pentose phosphate as strongly enriched (Fig. 3a).

To assess the impact of *E. coli* glycolysis and oxidative phosphorylation on *P. aeruginosa* growth, we co-cultured *P. aeruginosa* with the core glycolysis and oxidative phosphorylation pathway mutants of *E. coli*, *Δpgi* and *ΔatpC*, respectively. In aerobic cultures using LB plus 4% glucose, wild-type *E. coli* BW25113 reduced *P. aeruginosa* CFUs by >1,000 fold, while *Δpgi* and *ΔatpC* mutants were unable to inhibit *P. aeruginosa* growth significantly (Fig. 3b). This is in line with the fermentation efficiency of the *Δpgi* and *ΔatpC* strains, which was severely compromised with no lactic acid and reduced acetic acid (>2 fold decrease) production and deficient acidification (pH > 5.5) of the liquid bacterial culture (Fig. 3c). In aerobic conditions lactic acid production is very low compared to acetic acid production, but none of the mixed acid fermentation mutants, *Δpta*, *ΔadhE* or *ΔldhA*, could abolish production of lactic acid and reduce acetic acid production at the same time (Fig. 3c). Accordingly, these mutants retained their ability to inhibit *P. aeruginosa* aerobically (Fig. 3b). On the other hand, the *Δpgi* and *ΔatpC* strains abolish lactic acid and reduce acetic acid production, and these mutants are the only ones unable to inhibit *P. aeruginosa* (Fig. 3b,c).

Because the environment in the mammalian gut is anaerobic and the fermentation process towards lactic acid production is much more efficient, we further tested this pathway anaerobically. As under aerobic conditions, the core metabolism *E. coli* mutants *Δpgi* and *ΔatpC* were unable to inhibit *P. aeruginosa* growth, acidify culture media and produce lactic acid in anaerobic cultures (Fig. 3d,e). Also *E. coli ΔldhA* and *ΔadhE* mutants exhibited significantly reduced lactic acid production [$P < 0.001$] (Fig. 3e) and an impaired ability to inhibit *P. aeruginosa* in an anaerobic culture (Fig. 3d). Thus, lactic acid production is crucial, while acetic acid production is helpful, in inhibiting *P. aeruginosa* growth either aerobically or anaerobically.

Lactic acid and acetic acid can inhibit *P. aeruginosa* growth and virulence. Supplementation of the *E. coli* mixed-acid fermentation products acetic acid and lactic acid have been reported to act as antimicrobials against *P. aeruginosa*[35–37]. We validated the role of these two metabolites in inhibiting *P. aeruginosa* growth at pH 5. Acidic pH of <5 is observed in an *E. coli* culture in the presence of sugars in either aerobic or anaerobic conditions (Fig. 3c,e). A concentration of 10 mM or more of acetic acid, which can be produced by *E. coli* in an aerobic

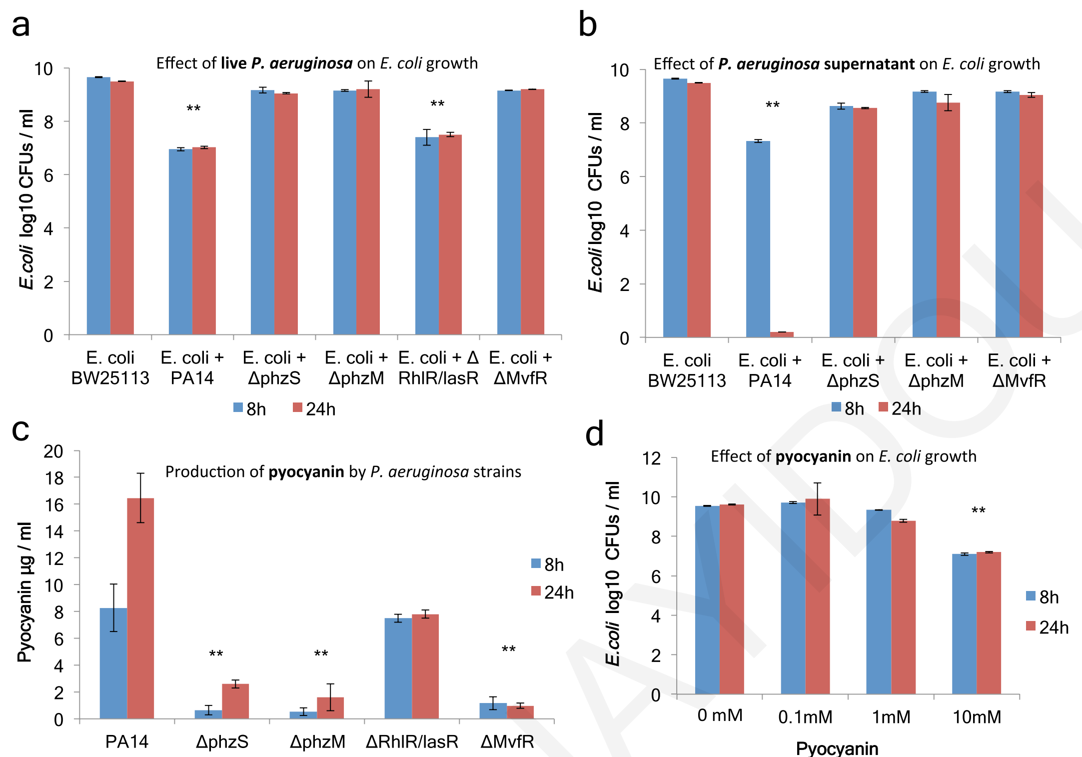

Figure 5. *P. aeruginosa* toxin pyocyanin inhibits *E. coli* growth in culture. (**a**) CFUs of *E. coli* BW25113 in co-culture with wild-type PA14 vs. isogenic QS mutants at 8 and 24 hours [n = 6]. (**b**) CFUs of *E. coli* BW25113 in 1:1 LB to LB supernatant of PA14 vs. isogenic QS mutants at 8 and 24 hours [n = 6]. (**c**) Pyocyanin concentration in LB cultures of wild-type PA14 vs. isogenic QS mutants at 8 and 24 hours [n = 6]. (**d**) CFUs of BW25113 at 8 and 24 hours in LB with supplemented pure pyocyanin [n = 6]. **p < 0.005. Error bars represent standard deviation of the mean.

liquid culture (Fig. 3c), abolished *P. aeruginosa* growth (Fig. 4a). Similarly, 10 mM or more of lactic acid, which can be produced by *E. coli* in an anaerobic culture (Fig. 3e), inhibited *P. aeruginosa* growth (Fig. 4b).

The inhibitory effect of *E. coli* lactic and acetic acid genes was further tested in animal models. In fly infection experiments, the *ΔldhA E. coli* mutant, specifically deficient in lactic acid production, and the core metabolism mutants *Δpgi* and *ΔatpC*, which are also unable to produce lactic acid aerobically or anaerobically, exhibited diminished ability to rescue flies infected with *P. aeruginosa* (Fig. 4c). In contrast, the *Δpta* and *ΔadhE* mutants, which cannot abolish lactic acid production in culture, rescued flies to the levels of the wild-type isogenic *E. coli* strain BW25113 (Fig. 4c). The same pattern was observed during co-infections in mice. We noticed that the *E. coli* mutant *ΔadhE* significantly rescued 60% of mice from lethality upon oral *Pseudomonas* infection in mice, comparable to the wild-type isogenic *E. coli* strain BW25113 that rescued 40% of mice, unlike the lactic-acid-defective strain *ΔldhA* that did not provide any significant rescue (Fig. 4d), suggesting that lactic acid production is the key for *E. coli* to inhibit *P. aeruginosa* in the host.

P. aeruginosa antagonizes _E. coli_ strains unable to ferment sugars to lactic acid. In the absence of added sugars in the culture media, *P. aeruginosa* inhibited *E. coli* growth rather than being inhibited by it (Fig. 5a,b). Screening for *P. aeruginosa* mutants implicated in this process, we identified the phenazine system and its known regulators (*phzS*, *phzM* and *mvfR*) as necessary for *E. coli* growth inhibition by >100 fold in culture (Fig. 5a,b). Pyocyanin, a redox-active secondary metabolite and a potent antibacterial, is produced and secreted by *P. aeruginosa* under the strict control of these quorum-sensing regulators. On the other hand the lasR/rhlR QS system regulators cannot fully control the expression of pyocyanin (Fig. 5c), and thus their mutation does not abolish the ability of *P. aeruginosa* to inhibit *E. coli* (Fig. 5a). Supplementation of 10 mM of pure pyocyanin was sufficient to inhibit *E. coli* in LB cultures to the same extent as in co-cultures with *P. aeruginosa* (Fig. 5a,d).

Moreover, the *E. coli* mutants, *Δpgi* and *Δldha*, which are deficient in glycolysis and lactic acid production respectively, were unable to inhibit *P. aeruginosa* growth in culture with and without supplementation of 4% glucose or sucrose (Suppl. Fig. 5a,b). Accordingly, we assessed the ability of *P. aeruginosa* to inhibit colonization by the *E. coli* mutants *Δpgi* and *Δldha*. Flies were inoculated with one or both species together and offered 2% or 4% glucose or sucrose as a necessary fly food nutrient. Co-inoculation of *Drosophila* with the *E. coli* glycolysis mutant *Δpgi* and *P. aeruginosa* showed reduced *E. coli* CFUs on day 2 and 5 compared to single inoculation with *E. coli Δpgi* (Fig. 6a,b). Moreover, flies mono-inoculated with *P. aeruginosa* or the *E. coli* lactic acid mutant *Δldha* could be colonized with an average of 4.1 (±0.5 SD) and 3.5 (±0.2 SD) $\log_{10}$ CFUs, respectively. Immediately after co-inoculation though *E. coli Δldha* CFUs were low, exhibiting an average of 1.9 (±0.2 SD) $\log_{10}$, while

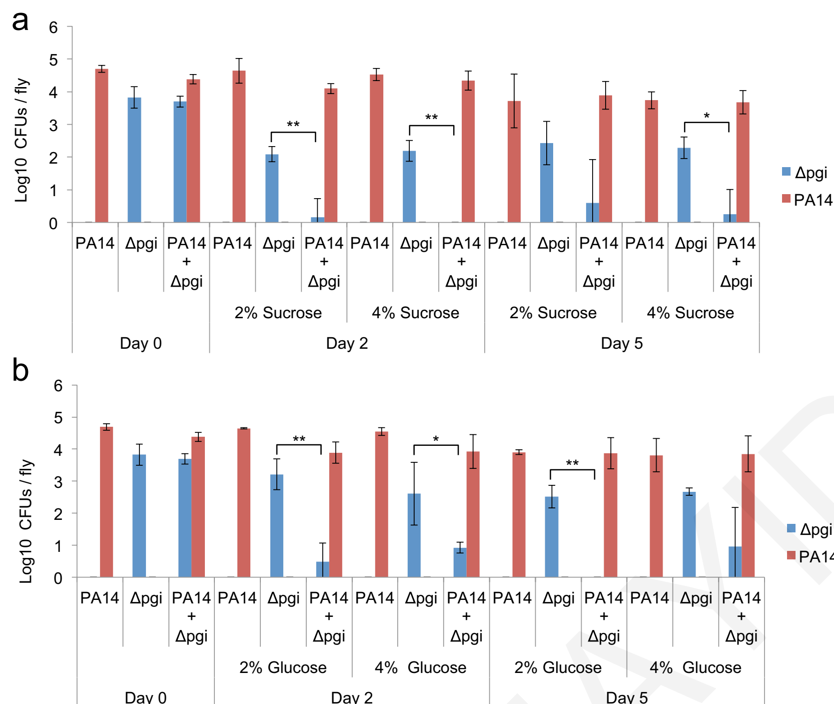

Figure 6. *P. aeruginosa* inhibits fermentation mutant *E. coli* in the *Drosophila* gut, despite dietary sucrose or glucose. (**a**,**b**) *Drosophila* intestine CFUs at 0, 2 and 5 days of flies orally infected with the *E. coli* BW25113 *Δpgi* core glycolysis mutant and/or PA14 [n = 6]. 2% or 4% sucrose (**a**) or glucose (**b**) was added in the infection medium and the fly food as a standard nutrient for the flies. $^{*}p < 0.05$, $^{**}p < 0.005$. Error bars represent standard deviation of the mean.

P. aeruginosa remained high at 3.8 (±1 SD) $\log_{10}$ CFUs. Thus with or without dietary sugars *P. aeruginosa* may inhibit gut colonization by *E. coli* mutants unable to ferment sugars into lactic acid.

Lactic and acetic acid production rather than nutritional input defines the interaction between *E. coli* and *P. aeruginosa* in the mouse gut. Diet is very important for the maintenance of a healthy microbiome and in shaping the intestinal immune response[38–40]. Our study shows that the interaction between *E. coli* and *P. aeruginosa* is shaped by the fermentation of sugars. Therefore we sought to investigate in mice the contribution of three nutritionally extreme diets: a protein-based, a vegetable fat-based and a carbohydrate-based diet. In mice on the carbohydrate-based diet the total sugar concentration (sucrose, glucose and fructose) in the faeces was 67.4 μg/ml, which was higher than any of the other diets, while it was only 18.7 μg/ml in the faeces of mice given *E. coli* orally (Suppl. Fig. 6a). This means that *E. coli* is consuming sugars in the mouse gut. Yet faecal lactic acid concentration was the highest in the fat-based diet group in the presence of *E. coli* (Suppl. Fig. 6b). Accordingly, *P. aeruginosa* CFUs were reduced by *E. coli* in mice fed with the vegetable fat-based diet (Fig. 7a), but not with the protein- or the carbohydrate-based diets (Fig. 7b,c). Similarly, mouse survival upon *P. aeruginosa* infection in immunosuppressed mice was the highest in mice fed the vegetable fat-based diet and co-inoculated with *E. coli*, as opposed to mice fed with the carbohydrate-based diet and co-inoculated with *E. coli* (Fig. 7d). However, the fat-based diet does not favour *E. coli* gut colonization, as the *E. coli* CFUs in the faeces are comparable between the carbohydrate- and the fat-based diets and lower than those of the protein-based diet (Fig. 8a).

To assess if faecal acetic and lactic acid production may indicate protection against *P. aeruginosa*, we correlated acid concentration in the faeces with the lethal time 50% (LT50) of the corresponding sets of mice. We found that lactic but not acetic acid levels alone correlate significantly and positively with survival to infection (Fig. 8b,c), while an index of normalized concentration values for acetic and lactic acid combined gave also a clear correlation with survival (Fig. 8d). We conclude that the standard balanced diet (Fig. 4d; Suppl. Fig. 3e) and the vegetable fat-based diet (Fig. 7a), rather than the carbohydrate-based diet (Fig. 7c) facilitate the inhibitory effect of *E. coli* on *P. aeruginosa*; and we suggest that, given the complexity of the mammalian intestinal environment, the metabolic output in acetic and lactic acid production rather than the dietary input is indicative of susceptibility to intestinal *P. aeruginosa* infection.

Discussion

Despite primarily observations on bacterial antagonism dating more than 100 years ago[41], there are not many cases supporting the model of one-pathogen-one-colonization-resistor, according to which specific bacterial strains protect the host against infection[42]. Known cases include the inhibitory effect of *Clostridium scindens* in resistance against *C. difficile* infection[43], the non-toxigenic *Bacteroides fragilis* resistance against the enterotoxigenic *Bacteroides fragilis*[44], the effect of *E. coli* O21:H+ against muscle atrophy due to infection, and the effect of

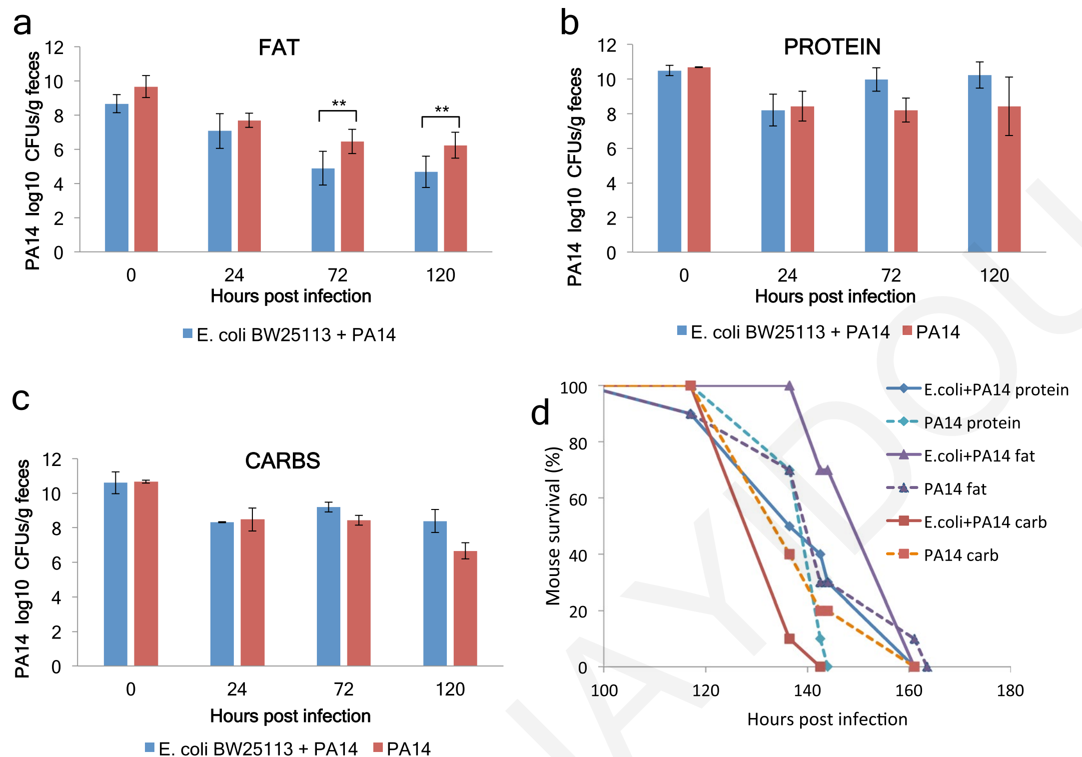

Figure 7. A fat-based diet, but not a carb- or protein-based diet enables *E. coli* to inhibit *P. aeruginosa* colonization and lethality in mice. (**a**) PA14 CFUs in faeces of immunocompromised mice fed on a fat- (**a**), protein- (**b**) or carbohydrate-based diet following inoculation with PA14 or co-inoculation with PA14 and *E. coli* BW25113 [n = 10]. $^{**}p < 0.005$. Error bars represent standard deviation of the mean. (**d**) Survival of immunocompromised mice kept on a fat-, protein-, or carb-based diet and infected with PA14 or PA14 plus *E. coli* BW25113 [n = 10]. $p = 0.04$ for Fat PA14 + *E. coli* vs. Protein PA14 + *E. coli* and $p = 0.0001$ for Fat PA14 + *E. coli* vs. Carbs PA14 + *E. coli*.

E. coli EcN against intestinal pathogens[42]. Antagonistic interactions between a pathogenic and a non-pathogenic bacterial strain may include: (a) the direct inhibition of pathogen's growth, colonization or virulence by the non-pathogenic strain, or (b) the indirect effect of the non-pathogenic strain in inducing or supporting the host defence to infection. To establish the mode of interaction between *E. coli* and *P. aeruginosa* we first examined whether *Drosophila* can be efficiently colonized with these species. Sugar-based diets allow stable bacterial colonization with either *P. aeruginosa* or *E. coli* strains. When flies are infected with both species, *P. aeruginosa* colonization and mortality is significantly reduced or eliminated. Antagonism between *P. aeruginosa* and other species is nevertheless specific. Human oropharyngeal bacteria are predominantly gram-positive, such as the *Neisseria*, *Streptococcus*, *Staphylococcus* and *Actinomyces* species, differ from those of the human intestine, and tend to induce rather than antagonize *P. aeruginosa* virulence[45]. Moreover, peptidoglycan, which is abundant in gram-positive bacteria, can directly induce the virulence of *P. aeruginosa*[46]. Thus *E. coli*, as opposed to many gram-positive bacteria, might serve as a safer inhibitor of *P. aeruginosa* by inhibiting its growth without inducing its virulence.

P. aeruginosa usually affects hospitalized and immunocompromised individuals. It causes life-threatening burn wound and lung infections, but humans often carry *P. aeruginosa* asymptomatically in their intestines[47]. During *P. aeruginosa* intestinal colonization, a healthy host primarily deploys innate immune responses recruiting macrophages and monocytes in the gut and then adaptive immune cells, such as B lymphocytes, through the induction of pro-inflammatory cytokines to control infection[48]. In immunocompromised patients, however, *P. aeruginosa* may disrupt the intestinal epithelial barrier and translocate extraluminally, leading to sepsis and death[49]. Moreover, virulent *P. aeruginosa* may facilitate this process by subverting the innate immune responses upon infection[50]. Another reason for the benignity of *P. aeruginosa* in the healthy human gut may be the action of intestinal microbiota, which are part of the host defence to intestinal infection[51–53]. Previous studies describe the use of antibiotic cocktails that favour *P. aeruginosa* intestinal colonization by compromising resistance by the intestinal microbiota[54]. Accordingly, we show that antibiotic use in mice diminishes all the prevalent phyla, eradicates *E. coli*, and induces dysbiosis. Using a *Pseudomonas*-induced gut-derived sepsis model to investigate infection in mice that exhibit neutropenia, lymphopenia, as well as mucosal damage[55,56], we found that mice not given antibiotics mostly survived and were less colonized with *P. aeruginosa*, contrary to antibiotic-treated mice. In addition, reintroduction of the commensal microbes through a faecal culture of endogenous and potentially beneficial bacterial species was inefficient in improving mouse protection from lethality. Nevertheless, a high dose of the endogenous *E. coli* CD1 isolate in combination with faecal bacteria exhibited significant protection

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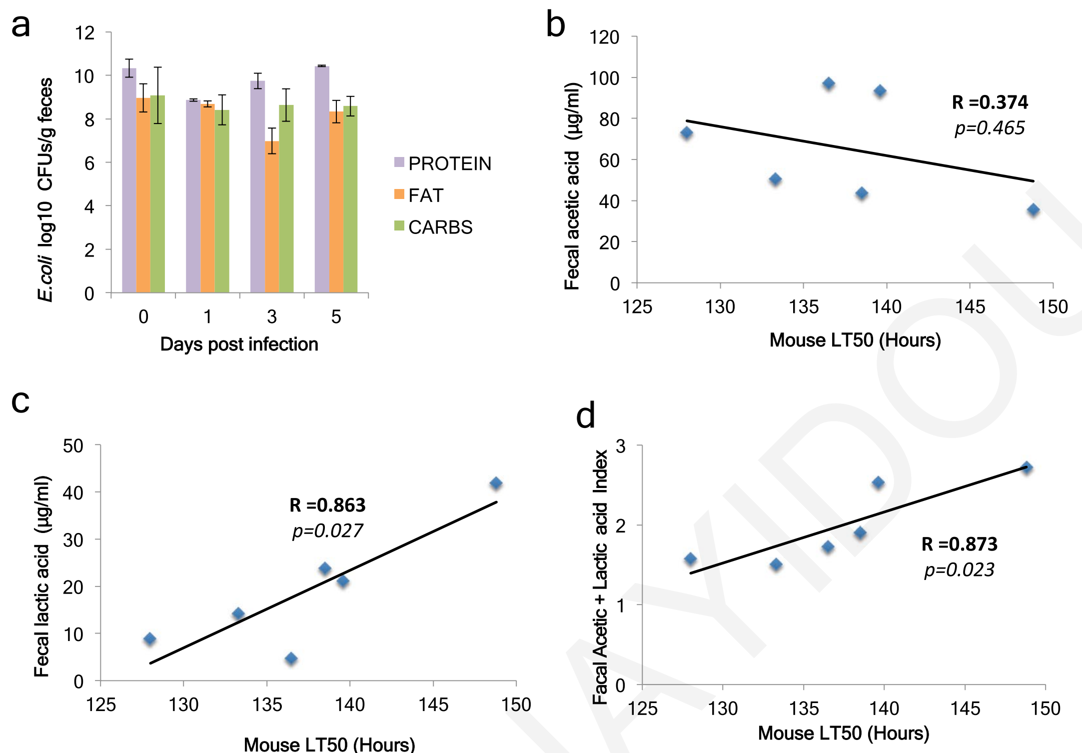

Figure 8. Faecal concentration of lactic and acetic acid correlates with mouse survival upon *P. aeruginosa* regardless of faecal *E. coli* levels. (**a**) *E. coli* CFUs in faeces of immunocompromised mice infected with PA14 and *E. coli* BW25113 and kept on a protein-, fat- or carbohydrate-based diet [n = 10]. (**b**,**c**) Correlation plots and Pearson correlation coefficient (R) of faecal acetic acid (**b**) and lactic acid (**c**) concentration against the lethal time 50% (LT50) of the corresponding mice for each of the 6 conditions (3 mouse diets × 2 types of infection). (**d**) A combinatorial index of normalized mouse faecal acetic and lactic acid concentration correlated with LT50.

against *P. aeruginosa*. We postulate a symbiotic adaptation of the mouse-isolated *E. coli* strain with the mouse gut environment and its microbiota in protecting the host.

Contrary to a previous study[27], we found that *E. coli* indole production had no effect on inhibiting the growth of *P. aeruginosa* in our experiments. This might be due to the inhibition of *E. coli* indole production by sugars added in our media or indole degradation via a higher induction of QS in the *P. aeruginosa* strain PA14[27,57]. Accordingly, we screened in an unbiased way and pinpointed *E. coli* glucose metabolism and fermentation mutants deficient in lactic and acetic acid production responsible for inhibiting *P. aeruginosa*. In antibiotic-treated mice, a similar trend was observed whereby the lactate-dehydrogenase-deficient *E. coli* mutant was unable to protect mice from *P. aeruginosa* infection and mortality. The anti-infective properties of lactic and acetic acid may be attributed to lowering the pH, but also to the permeabilization of the outer membrane of gram-negative bacteria[37]. On the other hand, *P. aeruginosa* produces many virulence factors regulated by QS, such as pyocyanin, which has bactericidal properties[58]. Accordingly, we notice that only strains of *P. aeruginosa* able to produce pyocyanin can inhibit *E. coli* unable to produce lactic or acetic acid due to the lack of sugars in the media. Interestingly, despite the fact that high sugar concentrations may inhibit *P. aeruginosa* QS[59], *P. aeruginosa* grown in sugar-supplemented media can still inhibit *E. coli* strains with mutated fermentation pathway genes. Thus depending on the concentration of *E. coli*'s acetic and lactic acid or *P. aeruginosa*'s pyocyanin, the antagonistic growth may be shifted towards one or the other species.

The role of diet has been extensively studied in response to gut microbiota and host physiology[60]. Hence we explored three different diets, based either on carbohydrates (corn starch and sucrose), fat (vegetable shortening) or protein (casein). Mice feeding on these diets exhibited complex features: First, the carbohydrate-based diet did not improve the ability of *E. coli* to inhibit *P. aeruginosa* colonization and concomitant mortality. This might be because this carbohydrate-based diet does not deliver a significant amount of free sugars in the mouse colon. While sugars are higher in the faeces of mice fed with a carbohydrate-based diet, they may be too low to have the anticipated impact on *E. coli*. Second, the protein-based diet sustains more *E. coli* than the other diets, yet this didn't translate into better inhibiting capacity against *P. aeruginosa*. This might be because casein inhibits or lacks the ability to fuel fermentation into lactic acid. Third, the vegetable fat-based diet, while not ideal for *E. coli* growth compared to the other diets, allows *E. coli* to produce more lactic acid that can inhibit *P. aeruginosa* growth. This is in line with evidence showing that unsaturated fat may benefit lactic acid bacteria in mice[61].

Lactic acid in the mouse faeces is much lower than the lowest inhibitory concentration tested in culture. Nevertheless, *E. coli* mutants defective in lactic acid production are also defective in inhibiting *P. aeruginosa* in the fly and mouse gut. The ability of any chemical to inhibit bacterial growth depends on the environment and

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thus additional factors (e.g. additional antimicrobials or lactic acid metabolism products) in the fly and mouse gut may boost the ability of lactic acid to inhibit *P. aeruginosa*. In addition, high sugar levels may be difficult to achieve by a high carbohydrate diet because sugar is readily absorbed in the small intestine, and *E. coli* and other commensals may use dietary fat more efficiently towards lactic acid production. Thus the metabolic output in the colon rather than the dietary input might better dictate the balance between and among bacterial species. Metabolic output is nevertheless a result of diet, microbiota composition and the host physiology acting in concert. Accordingly, faecal metabolomics might prove very helpful in predicting the outcome of bacterial interactions in the human colon and the risk for an infection.

Data Availability

All data will be available upon publication.

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25

Pseudomonas aeruginosa

Stavria Panayidou and Yiorgos Apidianakis

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25.1 Introduction

Classified in the family Pseudomonadaceae, order Pseudomonadales, class Gammaproteobacteria, the genus *Pseudomonas* comprises some of the most ubiquitous and diverse Gram-negative bacterial species in nature that are capable of utilizing a wide range of organic compounds and colonizing a variety of ecological niches. Among the members of this genus, *Pseudomonas aeruginosa* is remarkable for its capacity to inhabit diverse environments, including soil and water, and infect multiple organisms, such as insects, plants, and animals.[1–6] *P. aeruginosa* is an important opportunistic human pathogen inflicting predominantly burn, cystic fibrosis (CF), and otherwise immunocompromised patients. It is a frequent cause of nosocomial infections, being the most common pathogen isolated from patients hospitalized for longer than 1 week. One reason for its high prevalence is that it is foodborne—found, for example, in hospital water, food, and feeding tubes—and an efficient intestinal colonizer, especially upon antibiotic treatment and surgical stress.[7] Another reason is its high virulence repertoire, which includes biofilm formation and quorum-sensing controlled factors.[8,9] A third reason is its resistance to antibiotics.

The multifaceted pathogenicity of *P. aeruginosa* in humans necessitates the use of various models of infection and alternative model organisms. Due to ethical considerations and high cost of experimenting with vertebrate animals, invertebrates are widely used as alternative model hosts. In the following sections of this chapter, we describe the mammalian models that recapitulate pivotal aspects

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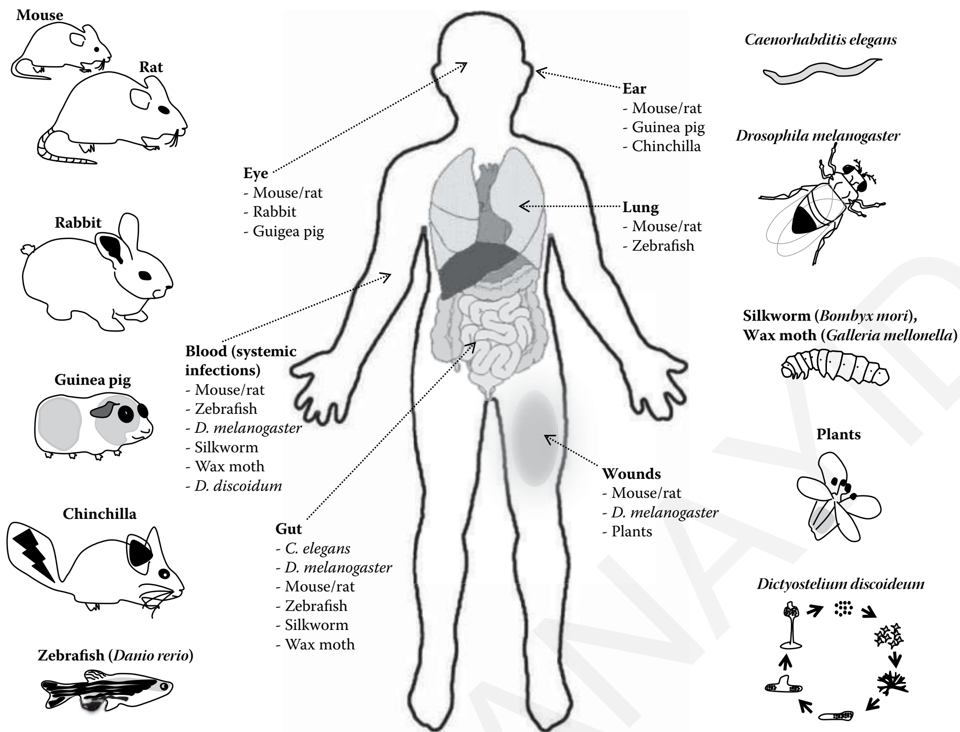

FIGURE 25.1 Model organisms recapitulating aspects of human blood, wound, lung, gut, eye, or ear infection with *P. aeruginosa*.

of severe *P. aeruginosa* pathogenicity, namely, burn and open wound, acute and persistent lung infection, and bacteremia, as well as less severe but potentially dangerous infections of the ear, eye, and intestinal tract. Zebrafish and invertebrate models also recapitulate aspects of wound, systemic, or intestinal/epithelial barrier infection. All established models, including those based on plants, are useful for assessing virulence, the efficacy of various treatments, and the role of host defense to infection (Figure 25.1 and Table 25.1).

25.2 Vertebrate Models

25.2.1 Cystic Fibrosis Mouse Models of Chronic Lung Infection

CF is an inherited disease of the secretory glands that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.[10] *CFTR* gene mutations prevent the ion channels of the lung and other tissues from moving salt and water into and out of cells. As a result, mucus accumulates in the lungs, trapping bacteria that cause chronic infections.[10,11] *P. aeruginosa* is a common cause of chronic CF lung infection that may persist for decades[12] and leads to mortality in the majority of the cases, due to progressive lung damage. In 1992, only 3 years after the identification of the *CFTR* gene, Snouwaert et al. generated the first CF mouse model.[10,13] Since then, several mouse models of CF and the *P. aeruginosa* lung infection have been developed.[14] Although none of them are ideal, mouse models may provide significant information about the CF pathogenesis and are essential for the preclinical assessment of new therapeutics.[14] A method that mimics very well the human chronic lung infection of CF disease is based on the introduction of *P. aeruginosa*-laden agar/agarose or alginate beads in the mouse lung by transtracheal injection. This model was first described by Cash et al. in 1979 using rats.[15] In this model, agarose beads act as artificial biofilms and protect bacteria from a direct neutrophil attack, facilitating

TABLE 25.1

P. aeruginosa Models of Infection and Indicative Findings Using Each Model

***P. aeruginosa* Infection Models**	**Indicative Findings Using the Model**
Vertebrate Models	
Cystic fibrosis mouse models of chronic lung infection	• Suitable model for testing anti-inflammatory compounds e.g., BIIL 284. • *P. aeruginosa* and *B. cenocepacia* interaction in the mouse lung induces bacterial virulence and concomitant inflammatory response.
Acute lung infection mouse models	• Increased expression of IL-6 is associated with edema formation and decreased lung function. • IL-17 facilitates neutrophil recruitment in the infected lung areas of *P. aeruginosa*-infected mice. • Immunosenescence leads to impaired neutrophil response in the lungs. • IL-27 is implicated in sepsis-induced immunosuppression. • A leukopenic mouse model was developed for testing novel drugs.
P. aeruginosa gut-derived sepsis mouse models	• The probiotic bacterium *B. longum* strain BB536 suppresses the intestinal colonization of *P. aeruginosa.* • IL-1 is critical during gut-derived sepsis. • The bacteriophage strain KPP10 decreases *P. aeruginosa* burden and inflammatory response in the infected mice. • Surgical stress induces *P. aeruginosa* PA-I lectin in the mouse intestine, causing lethal sepsis. • PA-I lectin and cytotoxic exoproducts compromise the intestinal barrier. • PA-I lectin is highly expressed during intestinal ischemia/reperfusion injury in mice and contributes to lethal sepsis. • Surgical hepatectomy leads to low phosphate levels in the mouse intestine, which is sensed by *P. aeruginosa*, which then, enhances its virulence causing gut-derived sepsis.
Burn-wound and open-wound sepsis mouse models	• *P. aeruginosa* strain PAO1 quorum-sensing mutants exhibit reduced virulence in this infection model. • Suitable model for testing new antibacterial agents and treatments. • Targeting of fragellin protein can be a promising approach for the treatment of *P. aeruginosa*-infected burns. • Negative-pressure wound therapy can prevent sepsis. • Low GstA4 expression in the muscle causes susceptibility to infection.
P. aeruginosa keratitis models (mice, rabbits, guinea pigs)	Studies in rabbits: • Numerous antimicrobial treatments against *P. aeruginosa* keratitis have been assessed. • The *P. aeruginosa* proteases, elastase B, and PASP contribute significantly to the pathogenesis of keratitis, whereas alkaline protease has a lesser contribution. Studies in mice: • LiCl and β-catenin promote host resistance against *P. aeruginosa* keratitis. • Mouse TREM-2 suppresses corneal inflammation and promotes resistance to *P. aeruginosa* infection. • MRP8/MRP14 signaling amplify the inflammatory responses and increase corneal susceptibility. • Extracellular matrix protein Lumican, surfactant protein SP-D, and chemokine CXCL10 have protective roles against *P. aeruginosa* keratitis. • *P. aeruginosa* proteases, PASP, and MucD contribute significantly to keratitis pathogenesis. Studies in guinea pigs: • Evaluation of *P. aeruginosa* virulence factors, antimicrobial drugs, treatments, and host defense in the course of infection.
Otitis media models (mice, rats, guinea pigs, and chinchilla)	• Evaluation of *P. aeruginosa* virulence factors, inflammatory responses, and treatments.
Zebrafish (*Danio rerio*) (injection or feeding)	• *P. aeruginosa* strains PA14 and PAO1 are pathogenic to zebrafish embryos. • Infection is influenced by the developmental stage of the host. • CFTR mediates resistance against *P. aeruginosa* infection.

(*Continued*)

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TABLE 25.1 (*Continued*)

P. aeruginosa Models of Infection and Indicative Findings Using Each Model

***P. aeruginosa* Infection Models**	**Indicative Findings Using the Model**
Invertebrate Models	
Drosophila melanogaster (feeding, wounding, or injection)	• Humoral and cellular innate immunity is important against infection. • PA14 escapes from the host defenses by suppressing the expression of antimicrobial peptides and muscle genes at the wound site. • Selection of virulence-attenuated mutants, e.g., KerV, which is a conserved virulence factor in Proteobacteria. • Expression of the human lactonase PON1 protects flies from *P. aeruginosa* infection. • CHD1 is important for fly intestinal resistance against *P. aeruginosa*. • JNK signaling pathway synergizes with Ras1 oncogene to induce stem-cell-mediated tumorigenesis and invasion/dissemination in the fly midgut and hindgut, respectively. • The RhlIR and LasIR quorum-sensing systems are important for the full virulence in orally infected flies.
Dictyostelium discoideum (feeding)	• The *rhl* quorum-sensing system is required for the full virulence of *P. aeruginosa* strain PAO1. • *P. aeruginosa* strain PA14 is more virulent than PAO1. • *trpD*, *pchH*, *and pchI* mutants are attenuated in virulence not only in *D. discoideum* but also in flies and mice.
Caenorhabditis elegans (feeding)	• *P. aeruginosa* pathogenesis (slow or fast killing) against *C. elegans* depends on the bacterial culture media. • The transcription factor DAF-19, the bZIP transcription factor *zip-2*, and the small organic molecule RPW-24 are important for *C. elegans* immune response against *P. aeruginosa* strain PA14. • HTS assays are developed for identifying novel antimicrobials.
Galleria mellonella (Wax Moth) (feeding or injection)	• Different *P. aeruginosa* strains exhibit different strategies of evading the immune system of *G. mellonella* larvae. • *P. aeruginosa* metalloproteinase elastase B stimulates the humoral immune responses in *G. mellonella*. • *G. mellonella* oral infection model can be useful in investigating *P. aeruginosa* virulence mechanisms.
Silkworm (*Bombyx mori*) (feeding or injection)	• Screening for *P. aeruginosa* virulence factors in the silkworm larvae. • The NO detoxification enzyme NO reductase is important for full virulence of *P. aeruginosa* in the silkworm. • PvdE and ExoS are important virulence factors for *P. aeruginosa* to cross epithelial barriers.
Plants	
• *Arabidopsis thaliana* • Sweet basil (injection)	• *P. aeruginosa* strains PA14 and PAO1 are pathogenic to *A. thaliana* and sweet basil. • The antimicrobial compound rosmarinic acid protects the sweet basil root only from *P. aeruginosa* quorum-sensing mutants unable to form biofilms. • The *P. aeruginosa* virulence factor pyocyanin inhibits the development of *A. thaliana* roots though ethylene-dependent signaling. • PA14 pathogenicity islands, PAPI-1, and PAPI-2 carry many genes, 11 of which are necessary for full virulence in both *A. thaliana* and mice. • Salicylic acid attenuates the virulence of PA14 against *A. thaliana* and *C. elegans*. • *P. aeruginosa*-secreted proteases activate a novel *A. thaliana*-immune signaling pathway. • *P. aeruginosa* alkaline protease AprA cleaves the bacterial fragellin monomers in order to prevent immune recognition in both plants and mammals

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the modeling of delayed bacterial clearance. As a consequence, there is a prolonged neutrophil influx into the lung as well as accumulation of cytokines that resembles the *Pseudomonas* lung infection seen in CF patients.[16] Neutrophils are the protagonists of the excessive inflammatory response observed due to bacterial infections in CF patients,[17] but their role in pathogenicity is not clear. Accordingly, many studies are exploring the effect of specific anti-inflammatory compounds, such as BIIL 284, an antagonist of the leukotriene B4 (LTB_4)-receptor.[18] LTB_4 is a product of activated neutrophils and macrophages, and once it makes a complex with its receptor, it triggers NF-κB-dependent inflammatory responses.[18] BIIL 284 had previously caused adverse pulmonary reactions when given to CF patients. Therefore, Döring et al. examined the effect of BIIL 284 treatment in mice infected intratracheally by injection with the *P. aeruginosa* strain PAO1 embedded in agar beads.[18] Interestingly, BIIL 284 treatment led to decreased numbers of neutrophils, and consequently increased bacterial numbers, in the mouse lungs.[18] These observations were accompanied with strong presence of bacteria in the blood of the treated mice, compared with the untreated animals, indicating the important role of neutrophils in controlling bacterial lung infection and preventing sepsis.[18] The data also show the importance of mouse infection models for testing anti-inflammatory drugs before further assessment in clinical trials. Although most studies are focused on a single pathogen each time, CF disease is more complicated and often includes a number of different pathogens. Accordingly, the agar beads model of chronic lung infection has also been used to investigate the interaction between strains of two different opportunistic pathogens, *P. aeruginosa* and *Burkholderia cenocepacia*, in the mouse lung.[8] This coinfection increases the inflammatory response, as compared to the single infections, without any increase in the bacterial load, indicating that *P. aeruginosa* interacts with other bacterial species to increase bacterial virulence.[8]

25.2.2 Acute Lung Infection Mouse Models

Acute microbial lung infection, which mimics the human acute bacterial pneumonia, can occur in mice upon exposure to infectious aerosols or directly by intranasal or intratracheal instillation.[19] Intranasal infection allows the spreading of the bacteria from the upper airways to the intestine and the lower airways.[20] This protocol has been widely used for *P. aeruginosa* virulence factor assessment. Nevertheless, intratracheal instillation delivers much more bacteria into the distal bronchi.[20] Both *P. aeruginosa* acute lung infection and intratracheal instillation murine models can be used to gain insights about the immune responses and the lung function of the infected animals.[21] In mice infected intratracheally with *P. aeruginosa*, there is a correlation among increased interleukin-6 (IL-6) expression, edema formation, and decreased lung function.[21] In addition, the proinflammatory cytokine IL-17 facilitates the recruitment of neutrophils in the infected lung areas of infected mice.[22] On the other hand, immunosenescence leads to impaired neutrophil response, as observed in aged versus young mice subjected to intratracheal infection.[23] Septic mice infected intratracheally lead to the induction of IL-27, which in turn induces immunosuppression.[24] Toward standardization of new therapeutic approaches against human *P. aeruginosa* lung infections, Lawrenz et al. proposed recently a leukopenic (cyclophosphamide-treated) mouse model of lung intratracheal instillation for therapeutic testing of novel drugs against multidrug resistant strains.[25]

25.2.3 *P. aeruginosa* Induced Gut-Derived Sepsis Mouse Models

P. aeruginosa-induced gut-derived sepsis models mimic the pathophysiology of humans, because they involve intestinal colonization, proliferation, and invasion of other host tissues.[26] In order to produce murine gut-derived sepsis, mice receive bacteria in their drinking water as well as antibiotics e.g., ampicillin and streptomycin for a few days. Antibiotics disrupt the intestinal flora of the mice enabling gut colonization with *P. aeruginosa*.[27] To facilitate translocation of *P. aeruginosa* away from the gut, the immunosuppressant cyclophosphamide is administered during infection.[27] Interestingly, intestinal colonization with *P. aeruginosa* is reduced by a major member of probiotic bacterial species, *Bifidobacterium longum* strain BB536, which inhibits *P. aeruginosa* adherence to the intestinal epithelial cells in a murine model of gut-derived sepsis, encouraging its further assessment as a probiotic for immunocompromised

patients.[28] Moreover, in IL-1-deficient mice, *P. aeruginosa* load and the inflammatory response are significantly higher in the liver during gut-derived sepsis.[29] This effect is reversed when mice are treated with the bacteriophage strain KPP10, as compared with the phage-untreated mice.[27]

Another way to induce lethal gut-derived sepsis is by surgical stress (30% hepatectomy).[30] Following surgical hepatectomy in mice, *P. aeruginosa* expresses PA-I lectin/adhesin in the intestine of the animals, indicating that pathogens may sense host stress and respond by expressing specific virulence effectors that promote lethal sepsis.[30] The PA-I lectin contributes to damaging the intestinal epithelium barrier by compromising enterocyte tight junctions.[31] Moreover, *P. aeruginosa* senses low phosphate (Pi) levels in the mouse intestine following surgical hepatectomy, promoting lethal gut-derived sepsis.[32] Similarly, mice subjected to intestinal ischemia/reperfusion injury exhibit *P. aeruginosa* PA-I lectin-dependent translocation from the cecum to other organs including liver, lung, and kidney causing lethal sepsis.[33] These, and other studies, suggest that *P. aeruginosa* exhibits enhanced virulence upon stress, surgery, and trauma, all of which may promote intestinal pathologies and systemic bacterial spreading.[7,30–33]

25.2.4 Burn- and Open-Wound Infection Mouse Models

The *P. aeruginosa*-infected burn-wound sepsis model is used to mimic the human burn wound sepsis.[9,34] In 1975, Stieritz and Holder developed a nonlethal thermal injury to examine the pathogenesis of *P. aeruginosa* infection by injecting viable bacteria into the burn skin area.[34] Injection in the burn area caused rapid sepsis,[34] systemic inflammatory response syndrome, and multiple organ dysfunction syndrome.[35] This model allows the investigation of the pathogenicity of various *P. aeruginosa* strains and the identification of virulence factors. For example, Rumbaugh et al. demonstrated that the single quorum-sensing mutants, *lasI*, *lasR*, *rhlI*, exhibited reduced virulence compared to the wild-type PAO1 strain, while the double-mutant *lasI rhlI* was even more attenuated in virulence, suggesting the important role of quorum sensing in virulence in this infection model.[9] Additionally, the burn-wound sepsis model is useful for testing antibacterial agents and evaluating treatments for *P. aeruginosa*-infected burn patients.[36]

P. aeruginosa fragellin is a structural component of flagella and a potent immunostimulant. Barnea et al. examined the effect of anti-fragellin subtype A monoclonal antibody (anti-fla-a) in a *P. aeruginosa*-infected burn-wound mouse sepsis model.[37] Anti-fla-a reduced the mortality and morbidity of the infected mice, showing that targeting fragellin protein can be a promising approach for the treatment of *P. aeruginosa*-infected burns.[37] Moreover, negative-pressure wound therapy may prevent sepsis and decrease mortality by inhibiting the invasion and proliferation of *P. aeruginosa* in the injured tissue of burn-wound septic mice.[38]

In addition to burn-wound, open wound infection models can be used to evaluate and treat infections in the absence of severe systemic stress caused by burns. It involves the removal of ~1 cm^2 of skin from the mouse back and the application of luminescent or GFP-expressing *P. aeruginosa* cells that can be followed longitudinally along with the assessment of mouse survival.[39,40] In a pivotal study, glutathione *S*-transferase A4 (GstA4), a detoxification enzyme against lipid peroxidation byproducts, was found downregulated in human and mouse muscles following burns, while low muscle expression postburn in humans predicted their susceptibility to infection.[41] Moreover open wound infection with *P. aeruginosa* of wild-type and GstA4 mutant mice shows that the mutant mice are more susceptible to infection, indicating the usefulness of the open-wound model to pinpoint genes relevant to both burn- and open-wound infections.[41]

25.2.5 *P. aeruginosa* Keratitis Models (Mice, Rabbits, and Guinea Pigs)

Keratitis is a disease of the cornea that can be due to the infection with various microbes, including bacteria.[42] The characteristics of bacterial keratitis include inflammation with concomitant pain and redness.[42] *P. aeruginosa* is a common cause of bacterial keratitis in humans,[42] but it can also infect the cornea of other mammals including mice, rabbits, and guinea pigs. For example, the guinea pig is a model for evaluating not only antimicrobial drugs, identifying treatments,[43–48] and studying the

pathogenesis of *P. aeruginosa* keratitis but also host defense.[49–51] Rabbits, on the other hand, have large eyes similar in size to those of humans and can be used to evaluate several parameters of the disease.[42] The strain most commonly used to model bacterial keratitis and the efficacy of multiple treatments against *P. aeruginosa* is the New Zealand white rabbit.[42,52–59] For example, Chen et al. demonstrated in two separate studies that lithium chloride (LiCl) and β-catenin promote host resistance against *P. aeruginosa* keratitis by reducing the inflammatory responses of the host and by decreasing the bacterial burden.[60,61]

Moreover, *P. aeruginosa* elastase B, protease PASP, MucD, and, to a lesser degree, alkaline protease contribute significantly to the pathogenesis of keratitis.[62–64] Furthermore, induction of the triggering receptor expressed on myeloid cells-2 (TREM-2) upon infection in cornea scrapes triggers PI3K/Akt signaling to confer resistance against *P. aeruginosa* infection.[65] In contrast, induction of myeloid-related protein-8 (MPR8) and MRP14 upon infection in cornea scrapes, despite promoting bacterial clearance, induces inflammation and concomitant susceptibility to infection.[66] Three additional proteins, the extracellular matrix protein Lumican, the Surfactant Protein D (SP-D), and the C-X-C motif chemokine 10 (CXCL10) protect against *P. aeruginosa* keratitis in mice.[67–69]

25.2.6 Otitis Media Models (Mice, Rats, Guinea Pigs, and Chinchilla)

Otitis media (OM) includes a group of inflammatory diseases of the middle ear that can be caused by various conditions including infections by pathogens. Several models have been developed in various animals, including mice, rats, guinea pigs, and chinchilla, to evaluate *P. aeruginosa* biofilm formation, virulence factors, and the role of inflammatory responses and ciprofloxacin-hydrocortisone treatments against OM.[70–75]

25.2.7 Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*) is an attractive vertebrate animal model for studying host–pathogen interactions.[5,76] One of its advantages is that, unlike invertebrates, it has an adaptive immune system similar to that of mammals, although most infection models are based on injecting embryos that only have innate immune system.[5,76] Moreover, zebrafish embryos are transparent, which allows the visualization of bacterial infections in real time by using microbes that express fluorescent proteins.[5,76] Rawls et al. took advantage of this transparency to monitor the motility defects of *P. aeruginosa* flagellar mutants within the intestine *in vivo* and in real time and to assess the impact on host immune responses.[77] Another important advantage of zebrafish model is the availability of a wide range of genetic tools that permit generation of ~200 progeny following a single mating.[5,76] Therefore, zebrafish models could help clarify important aspects of the host innate immunity upon bacterial infection. Live bacteria of the wild-type *P. aeruginosa* strains PA14 and PAO1 can kill injected embryos, while quorum sensing and type three secretion system mutants are attenuated in virulence.[76] However, strains significantly attenuated in virulence in late developmental stages are highly lethal in early embryos that lack pivotal innate immunity mechanisms.[76] Furthermore, embryos with reduced *CFTR* gene expression (Cftr morphants) produce less reactive oxygen species (ROS) in their phagocytes and sustain more bacteria during infection.[78] ROS production by phagocytes is known as respiratory burst response and is an important host defense mechanism. Thus, Cftr morphants indicate a connection between the CFTR function and the innate immune response.[78]

25.3 Invertebrate Models

25.3.1 *Drosophila melanogaster*

Drosophila melanogaster (the fruit fly), despite its small size (~2 mm in length), is a great invertebrate model organism that adequately reflects some aspects of the mammalian pathogenesis of infection.[79,80] Its short life cycle and easy rearing allows the production of up to ~50 adult progeny per female fly within

2 weeks, which facilitates the large-scale *in vivo* screening of bacterial mutants. Many human bacterial, fungal, and viral infections can be studied in *Drosophila*.[81] Notwithstanding the lack of adaptive immunity as we know it in mammals, *Drosophila* has similar innate immunity, disease-related signaling pathways, and cellular types to those of mammals. Thus, it is a good model for studying the pathogenicity of microbial infections, including those caused by *P. aeruginosa*.[79,80,82]

There are three most common methods to infect *Drosophila* with *P. aeruginosa*[2,82,83]: (1) the feeding method involves mixing of bacteria with the fly food, which causes intestinal colonization and fly lethality within a few days; (2) the thoracic or abdominal needle pricking infection, that is, an injury being caused using a tungsten needle dipped into a bacterial suspension. Accordingly, bacteria are introduced locally at the wound site and later on spread systemically, killing the flies within 2–4 days; and (3) the injector pumping, which appears similar to the pricking method, but is actually a method of systemic infection and involves the injection of a controlled dose of bacteria directly into the fly hemocoel with a thin glass capillary tip.[83]

The latter two methods have been used to screen *D. melanogaster* for virulence-related mutants of the *P. aeruginosa* strain PA14, for example the virulence-attenuating factor *hudA*[84] and the hypothetical methyltransferase KerV, which is conserved among Proteobacteria.[85] Moreover, NF-κB and JNK signaling pathways are important for flies to resist *P. aeruginosa* infection,[86] although highly virulent *P. aeruginosa* escapes host defenses by suppressing or evading the induction by these pathways that would normally activate antimicrobial peptides systemically and muscle genes at the wound site.[40,87] In addition, transgenic expression of the human lactonase paraoxonase-1 (PON1) in flies protects them from *P. aeruginosa* wound infection by interfering with the bacterial quorum sensing.[88] Thus, human innate immunity factors such as PON1 can be introduced and studied in *Drosophila*.[89]

Using the oral infection model, which recapitulates intestinal colonization and systemic dissemination of *P. aeruginosa*, new aspects of bacterial quorum sensing and intestinal pathology have been revealed. For example, the *Drosophila* chromatin remodeling factor chromo helicase domain protein 1 (CHD1) contributes to fly intestinal resistance to *P. aeruginosa* infection[90] and the quorum-sensing factor *rhlR* contributes to circumvent the fly cellular immune response when bacteria escape the intestine and spread systemically.[91] Actually, both RhlIR and LasIR quorum-sensing systems are required for full virulence in orally infected flies.[92] In addition, intestinal *P. aeruginosa* senses Gram-positive bacterial peptidoglycan to enhance its quorum-sensing-mediated virulence.[93] Strikingly, intestinal *P. aeruginosa* and the quorum-sensing-produced virulence factor pyocyanin induce intestinal stem-cell-mediated regeneration, which facilitates tumorigenesis in the presence of oncogenes or in the absence of tumor suppressor genes.[94] Moreover, the activation of the JNK innate immune signaling pathway in the adult *Drosophila* hindgut cells during *P. aeruginosa* infection synergizes with $Ras1^{V12}$ oncogene expression to induce enterocyte invasion and dissemination to distant sites.[95,96]

25.3.2 *Caenorhabditis elegans*

Caenorhabditis elegans is a small (~1 mm in length) transparent nematode living in the soil that feeds on bacteria. Its life cycle starts with the embryonic stage, followed by four larval stages (L1–L4) and adulthood. Its cellular simplicity and its small generation time facilitates screens related to human pathogens,[97] including *P. aeruginosa*, for the identification of virulence-related genes,[98] host defense factors,[99–101] and antimicrobials.[102,103]

P. aeruginosa can cause different pathologies in *C. elegans* depending on the culture media it grows in.[104–106] *C. elegans* dies slower when exposed to *P. aeruginosa* strain PA14 grown on nematode growth (NG) media due to the accumulation of the bacteria in the gut of the worms.[104] This is known as "slow killing assay" because *C. elegans* succumbs after a few days.[104] In contrast, the nematode dies within a few hours when PA14 is cultured in media of high osmotic strength. This is referred to as "fast killing assay," according to which worms die as a result of diffusible bacterial toxins in their food rather than bacterial growth within them.[105] *P. aeruginosa* strain PAO1 quickly paralyzes and then kills *C. elegans* by using hydrogen cyanide, a poison that could also inflict tissue damage in cystic fibrosis patients.[107] Additionally, the digestive tubes of nematodes fed on PAO1 grown in low-phosphate media become red

before they die.[106] This phenomenon named "red death" occurs due to the activation of three systems: the phosphate signaling (PhoB), the MvfR-PQS quorum-sensing system, and the pyoverdine iron acquisition system.[106] Recently, Kirienko et al. established a liquid-based killing assay to show that pyoverdine causes hypoxia-related toxicity to *C. elegans* and that pyoverdine production by the PA14 strain is necessary for killing the worms.[108]

Regarding host defense, many factors important for *C. elegans* innate immunity against *P. aeruginosa* were identified within the last decade, including DAF-19, the ortholog of the human RFX, *zip-2*, a bZIP transcription factor and a small organic molecule, 2N(3chloro-4methylphenyl)-quinazoline-2,4diamine (or RPW-24).[99–101] Moreover, Conery et al. established a high-throughput screening (HTS) protocol in *C. elegans* for the identification of novel anti-infectives against *P. aeruginosa*.[102] Similarly, Zhou et al. developed an HTS assay for secondary metabolites of endophytic fungi using extracts of medicinal plants associated with these fungi to identify bioactive molecules that prolong the survival of *C. elegans* after *P. aeruginosa* infection.[103] With the caveat that HTS hits may not be validated in other systems and that extracts do not provide information on specific chemicals, such studies might serve as a starting point for the discovery of novel therapeutics.

25.3.3 *Dictyostelium discoideum*

Dictyostelium discoideum is a slime mould[109] with two remarkable multistage life cycles.[110,111] One is the asexual cycle, known as social cycle, characterized by the formation of fruiting bodies that release spores.[110,112] Spores give rise to haploid amoebae, which need to feed on bacteria to undergo mitosis.[110] Starving amoebas aggregate, forming new fruiting bodies. If starvation is combined with darkness and humidity, the sexual cycle starts with the fusion of two haploid amoebas of the opposite mate types, which attract and cannibalize surrounding cells forming a macrocyst that releases recombined new amoebas.[110,113] *Dictyostelium* cells naturally live in forest soil, and, by obligingly feeding on bacteria, they can be a natural host of pathogenic bacteria. Thus, they can serve as a great model organism for studying the mechanisms of bacteria–phagocyte interaction. Indeed, *D. discoideum* has been used to investigate the virulence of many human bacterial pathogens, including *P. aeruginosa*.[114] For example, the wild-type *P. aeruginosa* strain PAO1 inhibits *D. discoideum* growth, while *rhl* quorum-sensing system is required for full virulence.[115] Interestingly, the *P. aeruginosa* strain PA14 is more virulent than PAO1 against *D. discoideum*.[116] This is probably because 169 genes are differentially expressed between the 2 strains.[116] Of note, a random mutagenesis screen of the *P. aeruginosa* strain 22D10 identified anthranilate phosphoribosyltransferase gene *trpD* to be important for quorum-sensing function and the siderophore pyochelin genes *pchH* and *pchI* for the induction of the type III secretion system. Importantly, *trpD*, *pchH*, and *pchI* mutants are also attenuated in virulence in the *Drosophila* pricking and feeding assays and the mouse lung acute infection assay.[117]

25.3.4 *Galleria mellonella* (Wax Moth)

The greater wax moth, the lepidopteran *Galleria mellonella*, is a widely used model host for investigating microbial pathogenesis.[118,119] Similar to other insects, its life cycle progresses from egg to larva, pupa, and, finally, adult (moth).[119] The larvae of *G. mellonella* are relatively large in size (1–3 cm), facilitating the injection of bacterial pathogens and antimicrobial compounds.[118,119] An additional asset compared to other invertebrate model hosts is that it survives at the physiologic mammalian temperature (37°C), which favors the growth of many human pathogens.[3]

While *G. mellonella* can be infected orally by feeding, most infection studies use the injection method.[118–120] For example, Andrejko et al. have shown that different strains of *P. aeruginosa*, including two clinical isolates induce innate immunity genes to different extents when injected into hemolymph.[121] Additional data show that injected *P. aeruginosa* escapes the cellular immune responses of *G. mellonella* larvae by causing destruction of their hemocytes.[122] The virulence factors responsible for this strategy are still unknown. However, other studies implicate the *P. aeruginosa* metalloproteinase elastase B in virulence and counteraction of *G. mellonella* immune responses.[123,124]

25.3.5 *Bombyx mori* (Silkworm)

The Silkworm, *Bombyx mori*, is the best-known species of the lepidopteran superfamily Bombycoidea. The life cycle of this insect mainly consists of four developmental stages: egg, larva, pupa, and adult.[125] Numerous immunological, microbiological, and pharmacological studies of many pathogens have been carried out using this simple model showing, for example, that the overactivation of innate immunity in silkworms induces tissue damage followed by host death, resembling sepsis-induced multiorgan failure in humans.[126] Moreover, *P. aeruginosa* mutants and toxins, namely, pyocyanin, gacA, superoxide dismutase (sodB and sodM), nitric oxide (NO)-detoxification enzymes (NO reductase and flavohemoglobin), exotoxin A, and pyoverdine pvdE, have been assessed for virulence against silkworm larvae.[127–133] Among those, pvdE induces ExoS production, which is a bifunctional protein with GAP and ADP-ribosyltransferase activity that facilitates the translocation of the bacteria from the lumen to the hemolymph.[132] Interestingly, the ADP-ribosyltransferase activity of ExoS acts on mouse lung pneumocytes to disrupt the pulmonary–vascular barrier during *P. aeruginosa* acute pneumonia, leading to bacterial dissemination.[134]

25.4 Plants

Arabidopsis thaliana is a popular model plant due to its small size, its short life cycle of only 6 weeks—required for each seed to germinate into a mature plant and produce ~5000 new seeds—and the large number of mutant lines and genetic tools available.[135] It is also a model host for studying various aspects of infection, including host immune responses and microbial virulence strategies.[4] *P. aeruginosa* pathogenicity against *A. thaliana* involves various steps.[136] Infection starts with the syringe-mediated application of the bacteria on the leaf surface. The bacteria then attach and congregate and enter the plant tissues via stomatal openings.[136] Next, bacteria proliferate locally in the substomatal cavity and the intercellular space and destroy the local plant cells before dispersing systemically.[136] *P. aeruginosa* strains PA14 and PAO1 can infect the roots of both *A. thaliana* and sweet basil (*Ocimum basilium*), killing these plants within a week.[137] At the initial stage of infection, the two strains form biofilm structures on the root surfaces of the plants, which is reminiscent of the condition of the lungs of cystic fibrosis patients.[137] Biofilms confer antibiotic resistance and persistent pathogenicity to the organism. Accordingly, the antimicrobial compound rosmarinic acid, a multifunctional caffeic acid ester secreted from the sweet basil root, shows some *in vitro* activity only against *P. aeruginosa* strains unable to form biofilms.[137] Also, *A. thaliana* root development is inflicted by the multihost *P. aeruginosa* virulence factor pyocyanin, which induces the production of ROS and subsequent *A. thaliana* ethylene-dependent signaling.[138] The disaccharide trehalose of *P. aeruginosa* strain PA14 is a virulence factor that promotes pathogenesis only in *A. thaliana* and not in other hosts including mice.[139] However, He et al., demonstrated that PA14 carries two pathogenicity islands, PAPI-1 and PAPI-2, that harbor virulence genes, 11 of which are necessary for full virulence in both *A. thaliana* and in mice.[140] The majority of those genes are present in *P. aeruginosa* clinical isolates,[140] indicating that PA14 could survive in evolutionarily diverse hosts by using conserved functions. Accordingly, Starkey and Rahme have published infectivity protocols of *A. thaliana* and lettuce for screening *P. aeruginosa* bacterial strains to identify virulence factors potentially conserved for pathogenicity against other hosts.[141] A high through put (HTP) *P. aeruginosa*-infection system of *Arabidopsis* seedlings was also proposed by Gopalan and Ausubel for the discovery of potent anti-infective agents.[142] Furthermore, the *A. thaliana* pathogenicity model was used for the identification of host defense mechanisms and factors that repress *P. aeruginosa* virulence. For example, plant-derived salicylic acid, which is a phenolic metabolite, attenuates *P. aeruginosa* virulence against *A. thaliana* and *C. elegans* by downregulating the production of several virulence factors, including pyocyanin, protease, and elastase and by reducing biofilm formation.[143] Remarkably, Cheng et al., discovered a novel *A. thaliana* immune signaling pathway, namely, a mitogen-activated protein kinase (MAPK) cascade that is activated by proteases secreted by *P. aeruginosa*.[144] Nevertheless, *P. aeruginosa* has developed mechanisms to escape from the immune system of both plants and mammals. For example, *P. aeruginosa* alkaline protease AprA cleaves the bacterial fragellin monomers, which are ligands of pattern-recognition receptors in both plants and mammals, preventing the recognition and clearance of the bacteria from the host.[145]

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25.5 Conclusions

While all described models are useful, none is ideal. The main asset of mammalian models lies in the close recapitulation of the human disease, especially regarding host response. Nevertheless, zebrafish and invertebrate models also mimic, to a lesser degree, the pathophysiology of systemic, wound, or intestinal/barrier epithelial infections. All models, including plants, are able to elucidate aspects of host defense, including adaptive and innate immunity in the case of mammals and innate immunity in the case of all the rest. Importantly, all models are suitable for screening *P. aeruginosa* virulence factors. As different models may reflect different aspects of infections, involve different virulence factors and require different treatments may be relevant in each case, it is not clear if some models are always superior to the others. Therefore, it is valuable to use as many different models of infection as possible in order to generate the strongest evidence for the relevance of a *P. aeruginosa* gene, and to provide the soundest rationale for the implementation of an anti-*P. aeruginosa* measure.

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REVIEW
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Human pathogenic bacteria, fungi, and viruses in *Drosophila*

Disease modeling, lessons, and shortcomings

Stavria Panayidou, Eleni Ioannidou, and Yiorgos Apidianakis*

Department of Biological Sciences; University of Cyprus; Nicosia, Cyprus

Keywords: *Pseudomonas aeruginosa*, *Serratia marcescens*, *Vibrio cholerae*, *Lactobacillus plantarum*, *Francisella tularensis*, *Mycobacterium marinum*, *Salmonella* Typhimurium, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*

Drosophila has been the invertebrate model organism of choice for the study of innate immune responses during the past few decades. Many *Drosophila*–microbe interaction studies have helped to define innate immunity pathways, and significant effort has been made lately to decipher mechanisms of microbial pathogenesis. Here we catalog 68 bacterial, fungal, and viral species studied in flies, 43 of which are relevant to human health. We discuss studies of human pathogens in flies revealing not only the elicitation and avoidance of immune response but also mechanisms of tolerance, host tissue homeostasis, regeneration, and predisposition to cancer. Prominent among those is the emerging pattern of intestinal regeneration as a defense response induced by pathogenic and innocuous bacteria. Immunopathology mechanisms and many microbial virulence factors have been elucidated, but their relevance to human health conventionally necessitates validation in mammalian models of infection.

Introduction

We interact with microorganisms throughout our lives. Some microbes are beneficial for the human body, while others can be pathogenic. The skin and the mucosal surfaces are the primary sites of host–microbe interaction.[1,2] The intestinal mucosa is one of the largest interfaces of the human body and is heavily colonized by numerous bacterial species,[1,3] some of which protect the host by modulating immune responses to fight pathogens, while providing tolerance to non-pathogens.[3] Beneficial bacteria adhesion to the intestinal mucosa may prevent the attachment and compete for space and food with suspected pathogens, thus preventing the colonization and invasion of pathogenic bacteria.[4]

Nevertheless, many bacteria, viruses, and fungi can cause dangerous infections especially under conditions that favor their growth and survival. Pneumonia and diarrhea together are the third cause of death among children under 5 years of age worldwide, accounting for 2 million deaths per year.[5] Food- and waterborne pathogens can cause acute or chronic infections to most individuals, while immunocompromised individuals due to skin burn, cancer treatment, or HIV infection, are highly susceptible to opportunistic pathogens. Also genetically predisposed individuals are more susceptible to infection, because conditions such the inflammatory bowel disease and cystic fibrosis, can alter the microbiota composition and host defense promoting the colonization and invasion of pathogenic bacteria.

Here we list 68 microbial species that have been studied in flies (**Table 1**) and review some of the 43 human microbes that have been modeled in *Drosophila melanogaster* (**Fig. 1**), describing the lessons as well as the shortcomings in studying human microbes in flies. It appears that many human infectious agents can be effectively studied in *Drosophila*, in cases where the pathologies exhibited in flies reflect conserved aspects of human disease or physiology.

Due to space limitations we do not describe the significant work done in *Drosophila* with *Pseudomonas entomophila*, *Erwinia carotovora*, *Beauveria bassiana*, *Drosophila* viruses, and other non-human pathogens (**Table 1**), focusing instead on studies aiming to explore in depth human microbial pathogenesis.

D. melanogaster, a Simple Host for Studying Microbial Diseases

D. melanogaster is a simple model organism for studying diseases caused by a great number of bacteria, fungi, and viruses. It has a short generation time simpler but analogous organ structure compared with mammals, and can be expanded at low cost.[2,134] Despite simplicity, many *Drosophila* defense mechanisms are highly conserved in mammals.[135] NFκB, JNK, and JAK-STAT signaling pathways are critical regulators of the immune responses in both flies and mammals.[135] Similarly to mammals one of the first lines of the *Drosophila* defense against microbes is mediated by barrier epithelia and their responses.[136] Infected tissue homeostasis and regeneration are also part of the defense response.[39,137,138]

Systemically, *Drosophila* fights many microbes primarily via the production of conserved antimicrobial peptides by the fat body (an analog of the mammalian liver), by the deposition of melanin that traps microbes and via phagocytosis by the plasmatocytes, which are analogous to the mammalian macrophages.[135] Bacteria, fungi, and viruses induce Toll and Imd, the two highly conserved

*Correspondence to: Yiorgos Apidianakis; Email: apidiana@ucy.ac.cy
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Table 1. Microbes studied in *Drosophila melanogaster*

Microbes	Human diseases caused	Lessons from *Drosophila melanogaster*
Gram-negative bacteria		
**Burkholderia cepacia* complex (Bcc)	-Respiratory infections in immunocompromised patients[6]	-TNFα pathway might act against Bcc wound infections in humans.[7] -Melanization seem to reduce the ability of bacteria to grow (increase resistance) in flies, but it also reduces the tolerance of flies to Bcc infection, presumably because melanization induces immunopathology.[8]
Burkholderia thailandensis	-A low in virulence relative of *B. pseudomallei*[9]	-Highly pathogenic in wild type flies when injected or orally administered, despite the induction of antimicrobial peptides[9]
**Chlamydophila pneumoniae* **Chlamydia trachomatis*	-Lung carcinoma[10] -Trachoma[11] -Detrimental effects on female reproductive health[11]	-The conserved Tom complex-mediated host defenses show specificity against *C. caviae,* but not against *C. trachomatis*[12]
Enterobacter cloacae		-Infection induces peptide Edin in a Relish-dependent manner in adult flies[13]
Erwinia carotovora		-The Imd and JAK-STAT pathways control the immune responses in the gut. The latter contributes to stem cell proliferation and epithelial renewal.[14] -There is a conserved role of PGRPs in gut homeostasis in both mammals and flies.[15]
Escherichia coli laboratory strains (non-pathogenic, non-commensal)		-Non-pathogenic when injected into wild-type flies.[16]
**Francisella tularensis*	-Tularemia[17]	-*F. tularensis* uses common and host-specific virulence factors to proliferate within *Drosophila* and mammalian phagocytes.[17-21]
**Helicobacter pylori*	-Gastric ulcers and carcinoma[22]	-JNK, RTKs, and MLC are activated in response to CagA in a tissue context-dependent manner.[22-24]
**Legionella pneumophila*	-Legionnaire disease[25]	-Dot/Icm system and the pertinent secreted effectors of *L. pneumophila*, is pivotal in its pathogenicity in flies and humans. -Some bacterial effectors are required for full infectivity of *Drosophila* cells only in specific host genetic backgrounds.
**Mycobacterium abscessus*	-Localized tissue infections -Disseminated infections in immunodeficient patients[26]	-Induction of AMPs production in *Drosophila*[26]
**Mycobacterium fortuitum*	-Skin and soft tissue infections -Postsurgical wound infections -Endocarditis[27]	-CD36 family of proteins is required for mycobacterial infection.[28]
**Mycobacterium marinum*	-Skin infections[29] -Arthritis[29] -Osteomyelitis[29]	-Innate immunity and autophagy stimulants and anabolic and antimycobacterial drugs can be tested in flies against *M. marinum* and other mycobacterial infections.[30-33]
Mycobacterium smegmatis		-Malpighian tubules of *Drosophila* are epithelial tissues that sense microbial invasion[34] -ESCRT machinery may restrict the mycobacterial growth within the host cells[35]
Photorhabdus luminescens		-Induces the Imd pathway[36]
**Providencia species*	-Infect many organisms including humans[37]	-*Providencia* infects *Drosophila;* mechanisms unknown[37]
**Pseudomonas aeruginosa*	-Lethal infections in cystic fibrosis and burn wound patients.[38]	-*P. aeruginosa* modulates the local host defense responses in a tissue-dependent manner and may contribute to epithelial inflammation and cancer in genetically predisposed organisms.[39] -There is an inverse correlation between biofilm formation and acute virulence and the ability of other microbial species to enhance *P. aeruginosa* virulence.[40]
Pseudomonas entomophila		-Causes loss of gut integrity including the loss of stem cells and death.[41] -Induction of systemic expression of antimicrobial peptide genes in flies after oral infection[42]

Asterisk indicates human-related species that have been studied in flies.

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Table 1. Microbes studied in *Drosophila melanogaster* (continued)

Microbes	Human diseases caused	Lessons from *Drosophila melanogaster*
Gram-negative bacteria (continued)		
**Salmonella* Typhimurium	-Gastroenteritis[43]	-JNK and p38 MAP kinases may drive the humoral and the cellular innate immune response respectively against *S.* Typhimurium.[44-46] -The secreted effector protein AvrA may inhibit JNK to promote infection.[44]
**Serratia marcescens*	-Pneumonia[47] -Meningitis[47]	-*S. marcescens* may cause intestinal pathologies and concomitant lethality, in accordance to the propensity of bacteria to damage mammalian epithelia.[48-50] -While phagocytosis and NFκB pathway induction promotes host defense, JAK-STAT pathway-induced intestinal regeneration appears to exacerbate infection.[49]
Spiroplasma poulsonii (intracellular symbiont)		-Increases susceptibility of *Drosophila* to certain gram-negative pathogens[51]
**Vibrio cholerae*	-Cholera[52]	-Suppression of intestinal stem cell division is likely a virulence strategy of *V. cholerae* because accelerated epithelial regeneration may protect the host against *V. cholera*.[53] -The barrier-disrupting effects of cholera toxin may act in parallel with Cl^- secretion to drive the pathophysiology of cholera.[54]
Wolbachia (intracellular symbiont)		-Female *Wolbachia*-infected flies are more resistant to *B. bassiana* infection.[55] -Induces resistance to RNA virus infections in flies;[56] not via the siRNA pathway.[57] -The mechanisms of *Wolbachia*-mediated antiviral protection are independent of the mechanisms underlying antibacterial protection[58,59]
Xenorhabdus nematophila		-Induces the Imd pathway[36]
**Yersinia pseudotuberculosis*	-Yersiniosis[60]	-The virulence factor KerV is a possible target for anti-infective drug design.[61]
Gram-positive bacteria		
**Bacillus anthracis*	-Anthrax[62]	-Endocytic recycling and cell membrane cholesterol are targets of *B. anthracis* toxins in flies and probably in humans.[62]
**Bacillus cereus*	-Gastrointestinal and non-gastrointestinal infections[63]	-Host defense mechanisms are not defined[64]
Bacillus thuringiensis		-*M. sexta* larvae Aminopeptidase N is a receptor for the *B. thuringiensis* Cry1Ac1 toxin[65]
**Enterococcus faecalis*	-Nosocomial infections[66]	-*E. faecalis* shows exceptional similarities in natural colonization of *Drosophila* and humans, a property that places *Drosophila* in a suitable position to assess its quorum sensing factors that relate to pathogenicity.[66,67]
**Lactobacillus plantarum*	-Enhancement of the intestinal epithelium barrier function[68]	-Unlike pathogenic bacteria *L. plantarum* colonization is induced by PON1 and does not induce PGRP-LE mediated defense response. -It naturally colonizes, induces intestinal regeneration, and facilitates *Drosophila* development.[69]
**Listeria monocytogenes*	-Listeriosis[70]	-Genetic screens in *Drosophila* identify host autophagy and bacterial factors required for resistance and susceptibility to *L. monocytogenes* infection, as well as, the metabolic changes in the host during infection.[71,72]
Micrococcus luteus	-Meningitis -Pneumonia -Arthritis[73]	-*M. luteus* is NOT pathogenic in flies. Nevertheless its phagocytosis can be studied in *Drosophila*.[74]
**Staphylococcus aureus*	-Pneumonia[75] -Necrotizing fasciitis[75]	-*Drosophila* models of *S. aureus* infection show the interplay of peptidoglycan recognition and evasion of this recognition by D-alanylated wall teichoic acid bound to peptidoglycan.[76-78]
Staphylococcus xylosus		-*MyD88* mutant flies are more resistant to starvation and to *S. xylosus* intestinal infection than wild-type flies.[79]

Asterisk indicates human-related species that have been studied in flies.

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Table 1. Microbes studied in *Drosophila melanogaster* (continued)

Microbes	Human diseases caused	Lessons from *Drosophila melanogaster*
Gram-positive bacteria (continued)		
**Streptococcus pneumoniae*	-Pneumonia[80] -Meningitis[80]	-*Drosophila* phagocytes exhibit an immunological memory.[81] -Circadian rhythms modulate the *Drosophila* defense against *S. pneumoniae*.[82]
Fungi		
**Aspergillus fumigatus*	-Aspergillosis[83]	-Drug screens in immunocompromised flies against various strains of *A. fumigatus* can reveal the efficacy of combinatorial drug treatments.[84]
Beauveria bassiana		-Inhibits the activity of phenol oxidases, which are the main melanization enzymes[85] -Cold stress increases resistance to *B. bassiana* infection.[86] -Female *Wolbachia*-infected flies are more resistant to *B. bassiana* infection[55]
**Candida albicans* **Candida glabrata*	-Superficial and systemic infections[87]	-Toll-dependent defense responses contribute to resistance although to a different extent against systemic *C. albicans* and *C. glabrata*.[88] -SAP proteases of *C. albicans* compromise the intestinal barrier function and contribute to pathology.[87]
Candida silvativa		-The N-terminal part of the major phagocytic receptor, Eater, binds several microbes including *C. silvativa*[89]
**Cryptococcus neoformans*	-Meningoencephalitis[90]	-Alternative routes of infection reveal the existence of intestinal defense pathways other than Imd and Toll as critical for host defense.[91,92] -Host cell autophagy contributes to pathogenesis.[90]
**Cunninghamella bertholletiae*	-Invasive mucormycosis[93]	-*Drosophila* models of infection show that iron availability in the growth media and iron availability in the host affect the virulence of *C. bertholletiae* isolates.[93,94]
**Fusarium moniliforme*	-Infects fatally immunosuppressed hosts[95]	-Test of antifungal treatments[95] -Pathogenic when injected to wild-type flies.[16]
Metarhizium anisopliae		-The fungal peptide Destruxin A suppresses humoral immune responses in *Drosophila*.[96] -The proteolytic activity of *Metarhizium anisopliae* PR1A triggers the expression of *Drosomycin* in *psh*-dependent manner[97]
Pneumocystis *(P. murina*, *P.carinii*, and *P. jirovecii)*	-*P. jirovecii* cause pneumonia in humans -*P. murina* and *P. carinii* are rodent pathogens	-Toll-deficient flies are resistant to infection with *Pneumocystis* spp.[98]
**Rhizopus oryzae*	-Infects fatally immunosuppressed hosts[93]	-Tarcolimus and posaconazole show promise in combinatorial treatments.[99]
**Scedosporium apiospermum* **Scedosporium prolificans*	-Infect fatally immunosuppressed hosts[95]	-Antifungal drug testing in Toll-deficient flies[95]
Viruses		
Cricket paralysis virus (CrPV)		-CrPV increases and decreases respectively the host and viral mRNA translation during infection[100]
*Dengue virus (DENV)	-Dengue fever (dengue hemorrhagic fever and dengue shock syndrome)[101,102]	-An RNAi response is triggered by DENV to control infection[101] -Additional factors conserved between *Drosophila* and humans have been found to control infection and those could be further explored in mammals.[102]
Drosophila C virus (DCV)		-Identification of factors involved in different viral-life cycle stages.[103] -Infected flies induce the peptidoglycan receptor protein PGRP-SA and upregulate AMP encoding genes[104]
Drosophila X virus (DXV)		-Infection of flies leads in the upregulation AMP encoding genes[104]
*Epstein-Barr virus (EBV)	-Several cancers[105,106] -Autoimmune diseases[107]	-*Drosophila* is a model host system for identifying human genes, such as tumor suppressors that are targeted by BRLF1 and are relevant to EBV-mediated tumorigenesis.[105,106]
Flock house virus (FHV)		-Induces apoptosis of *Drosophila* Line-1 cells by depleting *Drosophila* Inhibitor-of-Apoptosis protein DIAP1.[108] -Viral siRNAs might cause FHV persistent infections.[109]

Asterisk indicates human-related species that have been studied in flies.

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Table 1. Microbes studied in *Drosophila melanogaster* (continued)

Microbes	Human diseases caused	Lessons from *Drosophila melanogaster*
Viruses (continued)		
*Hepatitis B virus (HBV)	-Hepatitis -Cirrhosis -Hepatocellular carcinoma[110]	-*Drosophila* S2 cells were used as an expression system for viral protein preparation[110]
*Human cytomegalovirus (HCMV)	-Birth defects[111]	-Viral protein expression in *Drosophila* blocks embryogenesis[111]
*Human immunodeficiency virus 1 (HIV-1)	-Acquired immunodeficiency syndrome (AIDS)	-Vpu inhibits Toll and induces JNK pathway, depending on the tissue in which it is expressed.[112,113]
*Influenza A virus	-Flu pandemics[114]	-Adaptation of the virus for growth in *Drosophila* cells facilitates the identification of host genes that affect viral replication and aberrant host cell programming.[115,116]
Nora virus		-Mainly found in the intestine of infected flies[117] -Infection is not affected by mutations in the RNAi, Toll, or JAK-STAT pathways[118] although these and other pathways are induced upon infection[119]
*SARS coronavirus (SARS-CoV)	-Atypical pneumonia[120,121]	-*Drosophila* transgenic models of SARS-CoV indicate genetic interactions of the viral apoptotic proteins 3a and M with cytochrome c and the AKT pathway, respectively.[120-122]
Sigma virus (SIGMAV)		-Induces expression of the peptidoglycan receptor protein genes PGRP-SB1 and PGRP-SD and some, but not all, AMP genes[104] -Toll and Imd signaling are not significantly induced by Sigma virus infection[104]
*Simian vacuolating virus 40 (SV40)	-Oncogenic properties[123]	-The interaction of tumor antigen ST with PF2A and the concomitant centromere duplication may drive oncogenesis by SV40.[123]
*Sindbis virus (SINV)	-Sindbis fever, arthralgia, and rush[124]	-NRAMP family proteins are used by the SINV alphavirus to enter *Drosophila* and mammalian cells.[124] -ERK pathway induction is pivotal for *Drosophila* and mosquito host intestinal defense.[125]
*Vaccinia virus (VACV)	-Used as a vaccine for smallpox prevention[126]	-Useful model for identifying cellular factors required for viral entry[127]
*Vesicular stomatitis virus (VSV)	-Oncolytic virus[128]	-Similarly to mammalian TLR7, Toll-7 induces autophagy to suppress VSV infection in an NFκB-independent manner.[129] -Toll-7 recognizes the viral capsid, as opposed to viral RNA recognition by the mammalian TLR7.[130]
*West Nile virus (WNV)	-Highly pathogenic: fever, meningitis, encephalitis[131,132]	-Non-coding WNV RNA can induce and suppress RNAi in *Drosophila* and mammals.[131,133]

Asterisk indicates human-related species that have been studied in flies.

NFκB pathways of *Drosophila,* as well as the highly conserved in mammals' JAK-STAT pathway.[135] Viruses that infect *Drosophila* may also induce RNA interference and autophagy.[139] The many studies that have established the paradigm of innate immunity in flies provide one framework in which to analyze host-pathogen interactions with the added dimensions of specific virulence factor, regeneration and tolerance mechanisms.[135,140-142]

Infections in flies enable the study of infected tissues and organs without the ethical concerns that accompany mammalian hosts. Moreover, flies are amenable to anti-infective treatments and a great number of genetic tools based on the *Drosophila* genome are now available.[2,134] Prominent among those is the ability to conditionally inactivate every single gene using fly strains expressing gene specific RNAi constructs.[138]

During the last years flies played a critical role in identifying virulence factors of various opportunistic pathogens.[16] Some microbes use to a large extent similar virulence mechanisms to infect flies and mammals, and many virulence factors effective against mammals are also responsible for pathogenicity in flies.[143,144] As a result, a big array of microbes has been studied in fruit flies, including many important human-related microbes (**Table 1**). We discuss the most extensively studied of the human pathogens in the following sections (**Fig. 1**).

Modeling Human Microbial Diseases in *D. melanogaster*

Human wound, systemic, and intestinal infections can be easily recapitulated in *Drosophila* by pricking, injecting, and feeding flies, respectively, with the pathogens of interest[145] (**Fig. 1**). The method of thoracic or abdominal needle pricking involves the use of a metal needle dipped into a bacterial suspension.[145] If flies are pricked in the thorax, wounding is primarily imposed to the thoracic cuticular epithelium and the underlying muscle.[137,145,146] Upon inoculation at the wound site, the bacteria may proliferate locally and disseminate throughout the body of the fly, leading to both local and systemic tissue damage and immune response.[137,145,146] A second method is the "injector pumping" that

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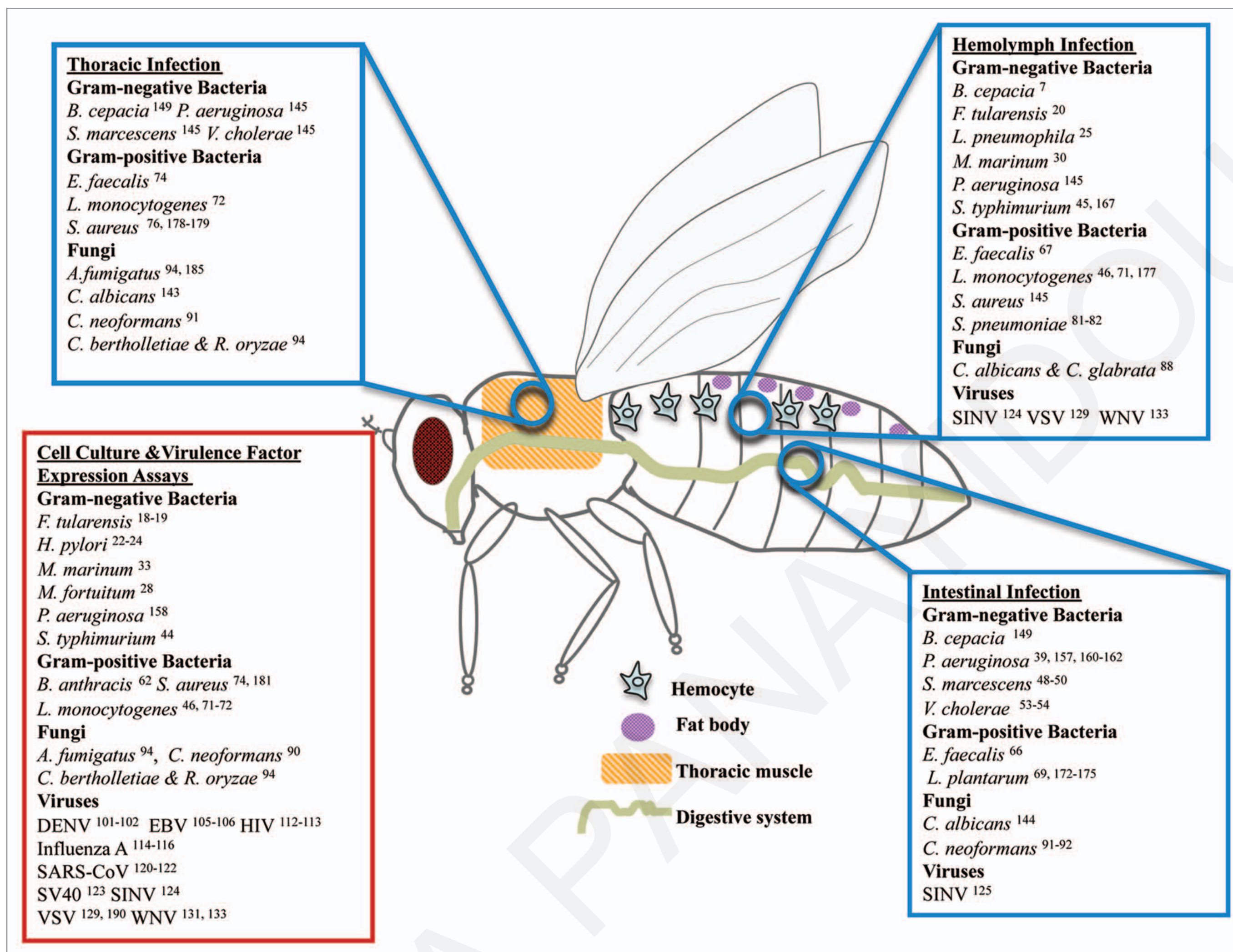

Figure 1. Human microbes extensively studied in *Drosophila*. Human microbes studied during their interaction with *Drosophila* in wound (thoracic pricking), systemic (hemolymph injection), or intestinal (feeding) infection assays. While depicted in adult flies, many hemolymph and intestinal infections are studied in larvae. In addition, microbial virulence factors have been expressed in live *Drosophila* tissues or *Drosophila* tissue culture cells have been studied upon infection with various human microbes.

produces primarily systemic inoculation by distributing microbes throughout the fly body.[145] Using this method adult flies or larvae can be easily injected with precise doses of the microbes of interest directly into the hemolymph, bypassing the wound site barrier.

Using *Drosophila* feeding assays to mimic mammalian intestinal infection various microbes can be introduced into the fly intestine.[145] This method provides the advantage, of the facile assessment of intestinal regeneration orchestrated by evolutionary conserved signaling pathways, including the JNK, Hippo, EGFR, and JAK-STAT signaling pathways.[147] Moreover, microbial genes can be individually studied by being expressed as transgenes in flies. This is a valuable technique necessary for studying human microbes that are unable to establish an infection in flies, expressing nonetheless virulence factors potentially harmful to both flies and mammals. Finally, infection of *Drosophila* hemocyte-like cell lines provides a means for high-throughput studies of microbe–immune cell interactions.

Many human bacterial, fungal, and viral pathogens have been studied in *Drosophila*. Some of them can be highly pathogenic in flies, while others are relatively harmless.[134,148] In the following sections we focus on *Drosophila* studies describing mechanisms of pathogenesis as potential targets against human pathogens.

Lessons from *Drosophila* Studies of Human Pathogens

Gram-negative and gram-positive bacteria, fungi, and viruses are grouped in separate subsections for systematic purposes.

Gram-negative bacteria

Burkholderia cepacia

Colonization with bacterial species of the *B. cepacia* complex (Bcc) is associated with serious respiratory infections in immunocompromised patients, such as cystic fibrosis and wounded individuals.[6] *B. cepacia* complex does not appear to kill *Drosophila* in

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feeding assays.[149] However, in wound infection (pricking) assays it is highly lethal and appropriate for screening Bcc mutants for virulence attenuation.[149] Mutant flies for *eiger*, the *Drosophila* TNFα homolog, die faster than wild type flies when injected with *B. cepacia*.[7] On the contrary, there is no increase in the mortality of flies mutant for melanization, although melanization-deficient flies bear on average more bacteria.[8]

Conclusion: TNFα pathway might act against Bcc wound infections in humans. Interestingly, melanization seems to reduce the ability of bacteria to grow (increases resistance) in flies, but also reduces the tolerance of flies to Bcc infection, presumably because melanization induces immunopathology.

Francisella tularensis

F. tularensis is the causative agent of tularemia which, is a zoonotic disease affecting many hosts including humans.[17] Most strains require biosafety level 3 handling due to the potential aerosol transmission. Flies and other arthropods, transmit *F. tularensis* to small mammals, such as rabbits.[150] *D. melanogaster* has been established as a good arthropod model for studying tularemia.[150,151] For example, out of 394 mutants assessed for defects in intracellular proliferation, 135 were defective is both *Drosophila* S2 cells and human macrophages.[18] Two virulence factors conserved in mammals, the PI4 kinase PI4KCA and the ubiquitin hydrolase USP22, are required for proliferation within the cytosol while a third, the ubiquitin ligase CDC27, is important for the escape of *F. tularensis* into the cytosol of the host cells.[19] In addition, 249 mutant strains of *F. tularensis* subsp. *novicida*, potentially relevant to mammalian cell pathogenesis, were tested in adult flies.[17] This subspecies is attenuated in virulence in mammals yet lethal to flies allowing experimentation in a reduced biosafety level environment. Twenty percent of the genes tested in mice also contributed to adult fly pathogenesis.[17] In a similar Transponson Site Hybridization (TraSH) screen the transcription factor oxyR and the DNA repair proteins uvrB, recB, and ruvC were found to contribute to virulence.[20] These virulence factors resist oxidative stress and counteract the melanization that *Drosophila* uses as an immune response to infection. On the other hand, *F. tularensis* subsp. *novicida* is very sensitive to the antimicrobial peptides produced by the Imd-regulated immune response of the infected flies,[20] despite the ability of *Francisella* lipid A and Kdo core but not of O-antigen to confer resistance against *Drosophila* antimicrobial peptides.[21]

Conclusion: *F. tularensis* uses common and many virulence factors to proliferate within *Drosophila* and mouse cells. Nevertheless, the factors required for virulence in adult flies might be different from those inferred from in vitro or studies, and further studies are necessary to validate their significance.

Helicobacter pylori

Helicobacter pylori is a causative agent of peptic ulcers, atrophic gastridis and gastric carcinoma.[152] Virulent strains can inject the CagA effector protein into the host cells.[22] Expression of this virulence factor in *Drosophila*, promotes apoptosis or tumorigenesis through the activation of the JNK signaling and the activation of receptor tyrosine kinase (RTK) pathway genes, such as Gab adapters.[22,23] Similarly, *Drosophila* transgenic models show that CagA activates myosin regulatory light chain (MLC), leading to the rapid disruption of epithelial integrity.[24]

Conclusion: JNK, RTKs, and MLC are activated in response to CagA in a tissue-dependent manner. Thus orthotopic activation of CagA in *Drosophila* stomach like tissues, for instance, midgut copper cells, might be recommended to validate these mechanisms of action.

Legionella pneumophila

L. pneumophila can cause severe pneumonia in humans called Legionnaire disease.[25] The bacteria direct the formation of their replication vacuole by injecting many effector proteins into the host cells via the Dot/Icm type IV secretion system,[153] a mechanism that is conserved in *Drosophila*.[25] In an RNA interference screen using *Drosophila* cells *Legionella* protein complex Cdc48/p97 was found necessary for the subcellular localization of bacterial effector proteins into the host cells.[153] Another screen, which combined bacterial mutagenesis with *Drosophila* cell RNA interference, uncovered the role in pathogenesis of bacterial effectors, previously considered as redundant for bacterial replication inside host cells.[154]

Conclusion: The Dot/Icm system and the pertinent secreted effectors of *L. pneumophila* are pivotal for pathogenicity in both flies and mammals. Importantly, some bacterial effectors are required for full infectivity in *Drosophila* cells only in specific host genetic backgrounds.

Mycobacterium marinum and Mycobacterium fortuitum

M. marinum, a close relative to *M. tuberculosis*, causes human skin infections that may spread deeper, resulting in arthritis or osteomyelitis.[29] Injection of only 5 colony forming units of *M. marinum* suffices to kill 50% of flies.[30] Infected flies undergo a "wasting" process characterized by hyperglycemia and the loss of metabolic stores, similarly to what happens in humans. This process is partially induced by the transcription factor FOXO, which nevertheless does not affect bacterial load.[155] Thus FOXO controls fly tolerance to *M. marinum* infection. In addition, infection with *M. marinum* does not induce the expression of antimicrobial peptides by *Drosophila*, as it is customary during other bacterial infections.[30] This means that flies—similarly to human lung cells infected with *M. tuberculosis*—fail to recognize and clear the bacteria or that bacteria actively suppress immune responses.[30] Strikingly, host cell autophagy activation is necessary process for successful antimycobacterial drug action in infected flies and mammalian macrophages.[31] And the highly conserved ubiquitin ligase *parkin* contributes to host defense against *Mycobacteria* and other intracellular pathogens in flies and mice.[32] Moreover, lysosomal enzyme β-hexosaminidase is sufficient to control *M. marinum* growth in S2 cells and mouse macrophages.[33] Furthermore, fly cell infection with *M. fortuitum,* which is also pathogenic to humans, is a useful model for the identification of conserved host factors, for example the CD36 family gene *peste*, that are required for *M. fortuitum* recognition and uptake by fly and human cells.[28]

Conclusion: Innate immunity and autophagy stimulants and anabolic and antimycobacterial drugs can be tested in flies against *M. marinum* and other mycobacterial infections.

Pseudomonas aeruginosa

P. aeruginosa is a major agent of lethal infections in cystic and burn wound patients.[38] Many of its virulence factors show exceptional conservation by contributing to pathogenesis in flies and mice.[156] *P. aeruginosa* redox-active phenazine pyocyanin induces *Drosophila* intestinal stem cells overproliferation as a defense response to infection, which nevertheless may lead to tumor formation in genetically predisposed flies.[39] In an oral infection model, in which the bacteria spread systemically to kill the fly, the quorum sensing regulator RhlR is required for full virulence.[157] In a wound infection model, transgenic flies expressing Paraoxonase 1 (PON1) are more resistant to *P. aeruginosa* wound infection, because PON1 can neutralize the quorum sensing regulator LasI.[158]

Interestingly, *P. aeruginosa* may interact with avirulent or beneficial bacteria in the fly alimentary canal to enhance its pathogenicity against *Drosophila*.[159] In the fly gut *P. aeruginosa* senses gram-positive bacteria peptidoglycan to induce its infectivity and virulence against eukaryotic and prokaryotic cells.[160] In addition, it may suppress the NFκB and JNK mediated innate immune response during wound infection but it may induce JNK signaling during intestinal infection to promote intestinal regeneration or tumor cell growth and dissemination.[38,161,162] *P. aeruginosa* actively limits the expression of *Drosophila* skeletal muscle genes at the site of wound infection and the expression of glutathione-S-transferase S1 (GstS1) in flies, a JNK-mediated response that is also conserved in mouse wound infections.[137] This wound site response is a resistance mechanism that inhibits bacterial growth and dissemination.[137] Interestingly, low expression levels of GstA4, the GstS1 homolog in mice and humans, proved later on to be a factor of susceptibility to wound infection in mice and humans.[163]

Recently, formation of *P. aeruginosa* biofilms was noticed upon infection in the *Drosophila* crop.[40] In this model biofilm formation correlates negatively with the virulence of the different strains. That is, mutants with decreased biofilm formation are significantly more virulent than hyperbiofilm strains, because the former disseminate more easily to the fly hemolymph and immune response is decreased, facilitating the progression of infection.[40]

The *P. aeruginosa*–fly model has still many aspects of infection to teach us because the *Drosophila* genotypic variation affects bacterial load and survival post-infection independently, suggesting that there are mechanisms of tolerance to infection which have not been studied.[164] Furthermore, evolutionary selection for traits that allow better survival of *Drosophila* to *P. aruginosa* infection reveal a correlation between organismal development and host defense, plus the importance of genes with dual involvement in developmental and immune pathways.[165] Thus pleiotropy might be a mechanism for the observed correlation.

Conclusion: *P. aeruginosa* modulates the local host defense responses in a tissue-dependent manner and may contribute to epithelial inflammation and cancer in genetically predisposed organisms. Moreover, *Drosophila* studies show that there is an inverse correlation between biofilm formation and acute virulence and the ability of other microbial species to enhance *P. aeruginosa* virulence.

Salmonella Typhimurium

S. Typhimurium is highly virulent due to its many virulence factors.[43] It can cause inflammatory diarrhea (gastroenteritis) in calves and humans and a typhoid-like disease in mice.[43] AvrA is among the effector proteins that *S.* Typhimurium secretes into the mammalian cells. Expression of AvrA in *Drosophila* suppresses apoptosis by inhibiting the JNK pathway, a conserved mechanism used by *S.* Typhimurium to restrict its elimination.[44] Consistently, AvrA was found suppressing innate immune response and inflammation in the mouse intestine.[166]

When injected into the hemocoel of *Drosophila*, *S.* Typhimurium is lethal[167] and similarly to most lethal infections it induces anorexia in flies. Anorexia in turn increases the fly's tolerance to *S.* Typhimurium infection.[168] Similarly, during *S.* Typhimurium infection, *eiger*, the only known TNF family member in the fly is required in the fat body to reduce the bacterial load via melanization.[45] *Eiger* mutant flies nevertheless survive the infection better because they are anorexic.[45] However, the relationship between diet restriction and host defense is not universal and should be evaluated on a pathogen-specific basis. Furthermore, the *Drosophila* p38 mitogen-activated protein (MAP) kinase (Dmp38b), a homolog of the mammalian p38 MAP kinase family, protects the host against *S.* Typhimurium, because it increases the phagocytic capacity of hemocytes.[46]

Conclusion: The JNK and the p38 MAP kinases may drive humoral and the cellular innate immune response respectively against *S.* Typhimurium, while the secreted effector protein AvrA may inhibit JNK to promote infection. Nevertheless, TNF pathway inhibition induces anorexia, which seems to contribute to host tolerance.

Serratia marcescens

S. marcescens is an entomopathogenic bacterium able to infect many hosts, including humans.[48] It is a significant cause of hospital-acquired infections with high mortality rates, especially in neonatal intensive care units as it may cause pneumonia, meningitis or other serious infections.[47] *Drosophila* intestinal infection with *S. marcescens* causes a local immune response but bacteria can also traverse the intestinal epithelium and gain access to the host's body cavity.[48] A genome-wide in vivo *Drosophila* RNAi screen using *S. marcescens* infected flies identified the JAK-STAT pathway as an important inducer of intestinal regeneration and a negative regulator of host defense to intestinal infection.[49] On the contrary, Imd/NFκB signaling activation upon infection induces host defense.[48,49] Moreover, bacteria that escape to the hemolymph are contained by phagocytes.[48] Ingested bacteria that translocate to the hemolymph are detected by the systemic humoral immune system only when phagocytosis is blocked.[48] Importantly, flies lacking the gene *subdued*, a member of the mammalian calcium-activated chloride channels-TMEM16 family, accumulate more bacteria and succumb faster than wild-type flies upon *S. marcescens* oral infection, indicating a role of this gene in the *Drosophila* resistance to infection.[50]

Conclusion: *S. marcescens* may cause intestinal pathologies and concomitant lethality, in accordance to the propensity of bacteria to damage mammalian epithelia. While phagocytosis and NFκB pathway induction promotes host defense, JAK-STAT

pathway-induced intestinal regeneration appears to exacerbate infection.

Vibrio cholerae

V. cholerae is the etiological agent of cholera, a life-threatening diarrheal disease. Humans are usually infected through ingestion of contaminated water, because this bacterium primarily exists in marine environments. *V. cholerae* polysaccharide (VPS)-dependent biofilm is highly activated upon entry into the arthropod intestine and is specifically required for colonization of the arthropod rectum.[169] Interestingly, intestinal infection of *D. melanogaster* with *V. cholera* mimics to a great extent the human disease cholera.[52]

KerV, a virulence factor conserved among pathogenic *Proteobacteria*, contributes to *V. cholerae* pathogenesis in *Drosophila*.[61] Furthermore, mutations in the pro-apoptotic Eiger/TNF signaling pathway increase the susceptibility of the fly to *V. cholerae* infection, suggesting that this pathway promotes host defense against this bacterium.[170] *V. cholerae* inhibits intestinal regeneration in infected flies, but Imd/NFκB pathway and *mustard* mutants counteract this inhibition, maintain higher levels of intestinal stem cell division, and survive better during *V. cholerae* infection.[53] Cholera toxin-driven inhibition of Rab11/exocyst-mediated trafficking of host proteins induces junctional damage, weight loss, and dye leakage in the *Drosophila* gut and other pathologies conserved in human intestinal epithelial cells, and ligated mouse ileal loops.[54]

Conclusion: Suppression of intestinal stem cell division is likely a virulence strategy of *V. cholerae* because accelerated epithelial regeneration may protect the host against *V. cholerae*. Also the barrier-disrupting effects of cholera toxin may act in parallel with Cl^- secretion to drive the pathophysiology of cholera.

Gram-positive bacteria

Bacillus anthracis

Bacillus anthracis is the etiological agent of anthrax, and can infect many mammals, including humans.[62] There are three factors secreted by this bacterium which contribute to its high virulence: the lethal factor (LF), theoedema factor (EF), and the protective antigen (PA).[62] PA contributes to the entrance of LF and EF into the host cells.[62] Expression of LF and EF in *Drosophila* during development, cooperatively inhibit the last step of endocytosis, namely endocytic recycling, by blocking the Rab11/Sec15 exocyst.[62] The role of LF and EF in endocytosis proved to be conserved in a human cell line.[62] Another *Bacillus anthracis*-secreted factor the hemolytic/cytolytic protein anthrolysin O binds and kills mouse and human macrophage-like, but not *Drosophila* S2 cells, because flies contain mainly ergosteror instead of cholesterol in their cell membranes.[171]

Conclusion: Endocytic recycling and cell membrane cholesterol are targets of *B. anthracis* toxins in flies and probably in humans.

Enterococcus faecalis

Enterococci, including *E. faecalis*, are commensal organisms of the gastrointestinal tract. Interestingly, *E. faecalis* appears to naturally colonize the *Drosophila* intestine and is the leading cause of many nosocomial infections. *E. faecalis* strains that express the virulence factor cytolysin are significantly more virulent to both flies and mammals.[66] Septic injury with *E. faecalis* activates phagocytosis in addition to the antimicrobial peptide production in *Drosophila*.[74] *E. faecalis* phagocytosis is regulated by the receptor Eater and is critical for the *Drosophila* host defense.[74] *E. faecalis* quorum regulatory system genes LrgAB and SprE, and bacteriocin EF1097 were found to contribute to infection toxicity in *Drosophila*.[67]

Conclusion: *E. faecalis* shows exceptional similarities in natural colonization of *Drosophila* and humans, a property that places *Drosophila* in a suitable position to assess its quorum sensing factors that relate to pathogenicity.

Lactobacillus plantarum

L. plantarum is a gram-positive commensal bacterium in humans suggested to protect the intestinal epithelium barrier function.[68] Recent studies demonstrate that *L. plantarum* can colonize germ-free *Drosophila* larval gut and remains associated with it long after the initial colonization.[69] A mechanism used by *L. plantarum* to establish itself in the gut is the recognition by PGRP-LE and the subsequent lack of inhibition of the Imd/NFκB pathway.[172] On the contrary, PGRP-LE senses entomopathogenic *Erwinia carotovora* and induces the Imd/NFκB pathway to defend the host from infection.[172]

Several *L. plantarum* strains stimulate larval development upon nutrient scarcity and adults emerge faster than in the germ-free flies.[69] Importantly, colonization with *L. plantarum* protects the fly from virulent *P. aeruginosa* and *S. marcescens* oral infection.[173] In addition, expression of human PON1, previously found to inhibit *P. aeruginosa* quorum sensing, is shown to increase *L. plantarum* colonization in the fly gut;[174] yet another mechanism to inhibit *P. aeruginosa* infection.

Interestingly, NADPH oxidase 1-dependent ROS generation and consequent cellular proliferation in intestinal stem cells are induced upon ingestion of *L. rhamnosus* and *L. plantarum* in mice and *Drosophila* respectively.[175] Although in disparate phylogenic clades, *L. rhamnosus* and *L. plantarum* seemingly have evolved the ability to induce cellular ROS and intestinal generation within their adapted host.

Conclusion: Unlike pathogenic bacteria, *L. plantarum* colonization is induced by PON1 and does not induce PGRP-LE mediated defense response. Due to its ability to naturally colonize, induce intestinal regeneration and facilitate larval development, *L. plantarum* studies in flies can be directly relevant to human health.

Listeria monocytogenes

L. monocytogenes, is an opportunistic anaerobic intracellular pathogen that causes listeriosis, which is presented by non-specific flu-like symptoms and gastroenteritis.[70] In a *Drosophila* cell culture RNAi screen many host factors were identified required for intracellular pathogenesis and factors that specifically affect access to the cytosol by *L. monocytogenes*.[176] Induction of autophagy in *Drosophila* requiring the autophagy-related factors Atg5 and Atg1 is crucial to prevent the intracellular growth of *L. monocytogenes* and promote host survival.[71] *Drosophila* genes conferring tolerance to infection were found to be specific to the different stages of infection.[8] For example, p38 MAPK-dependent phagocytic encapsulation of bacteria resulted in enlarged phagocytes that trap *L. monocytogenes* conferring tolerance to infection.[46]

L. monocytogenes virulence genes are expressed at 25 °C, and not only at temperatures higher than 30 °C as previously thought.[72] Moreover, similar bacterial genes, such as *actA* and *prfA*, are used in *Drosophila* and mammalian cells for the intracellular replication and cell to cell spreading of *L. monocytogenes*.[72] In addition, flies infected with *L. monocytogenes* exhibit a shift in their metabolism manifested primarily as changes in their lipid, carbohydrate, and amino acid levels.[177]

Conclusion: Genetic screens in *Drosophila* identify host autophagy, phagocytosis, and bacterial factors required for resistance and tolerance to *L. monocytogenes* infection, as well as the metabolic changes in the host during infection.

Staphylococcus aureus

S. aureus has been characterized as a nosocomial pathogen, but can also infect healthy individuals.[75] *S. aureus* infections can be life-threatening because they can cause pneumonia and necrotizing fasciitis.[75] *Drosophila* is used for studying the virulence determinants of *S. aureus* strains,[178,179] and the response to antibiotic treatment upon infection.[180] *Drosophila* infection by *S. aureus* can be controlled by phagocytosis mediated by the Eater receptor.[74] Toll pathway recognizes peptidoglycan from many gram-positive bacteria and contributes to resistance against *S. aureus*.[181] Wound infection of *Drosophila* with *S. aureus* shows that D-alanylation of wall teichoic acid alters peptidoglycan recognition by the Toll innate immune pathway[76] because D-alanylated wall teichoic acid binds covalently to peptidoglycan.[77,78]

Conclusion: *Drosophila* models of *S. aureus* infection show the interplay of peptidoglycan recognition and evasion of this recognition by D-alanylated wall teichoic acid bound to peptidoglycan.

Streptococcus pneumoniae

S. pneumoniae is a human pathogen that can cause serious pathologies, including community-acquired pneumonia and meningitis.[80] Flies injected with 3000 bacterial cells into the hemolymph are usually killed within 2 d.[81] However, flies challenged with a lethal dose after being primed with heat-killed bacteria resist infection.[81] Phagocyte activation is critical for immune priming.[81] Nevertheless, this long-lasting effect is not universal and needs to be evaluated individually for each microbial species.[182] Furthermore, flies infected with *S. pneumoniae* lose circadian rhythms several days before dying.[82] Consistently, flies lacking the central clock proteins *timeless* or *period* have higher sensitivity to *S. pneumoniae* but also to *L. monocytogenes* infection.[82] Interestingly, survival during a *L. monocytogenes* infection is determined by phagocytosis and melanization; while only phagocytosis determines survival during a *S. pneumoniae* infection.[183] A trade-off in phagocytosis is evident, because increased phagocytosis is beneficial to the host during *S. pneumoniae* infection but detrimental during *L. monocytogenes* infection.[183] This might be because the former is an extracellular and the latter an intracellular pathogen.

Conclusion: *Drosophila* phagocytes are protective and exhibit an immunological memory, while circadian rhythms modulate the *Drosophila* defense against *S. pneumoniae.* Whether similar mechanisms take place in humans will be important to explore.

Fungi

Aspergillus fumigatus

A. fumigatus is the major cause of invasive aspergillosis in immunocompromised individuals and adult flies, although other *Aspergillus* species are also pathogenic.[83] The virulence of *A. fumigatus* has a multifactorial nature.[184] In 1996 Lemaitre, Hoffmann, and collaborators found that the Toll pathway is required in *Drosophila* to respond to *A. fumigatus* infection.[185] While non-pathogenic to wild-type flies, this fungus is lethal to *Toll*-deficient flies,[83,185] which can also be used to screen for antifungal drugs combinatorially in vivo. For example, combinatorial treatments with voricanazole and terbinafine have been shown to have a synergistic effect against infection.[84] In addition, Toll-deficient flies have been used in combination with zebrafish to show that *A. fumigatus* secondary metabolites contribute to fungal virulence and phagocyte function respectively.[186]

Conclusion: Drug screens in immunocompromised flies against various strains of *A. fumigatus* can reveal the efficacy of combinatorial drug treatments.

Candida albicans and Candida glabrata

C. albicans is the predominant fungal pathogen in humans causing invasive infections and most commonly death in immunocompromised patients.[187] *C. albicans,* and to a lesser extend the microbiologically disctinct *Candida glabrata*, can cause superficial infections in several organs using tissue site-specific virulence factors, but also bloodstream infections in immunocompromised and inflammatory bowel diseases patients.[188] In immunocompromised patients the systemic dissemination is thought to occur from the gut to the bloodstream.[144]

The pathogenicity of *C. albicans* can be studied by systemically infecting Toll-deficient flies or by feeding wild-type *Drosophila* larvae, because in both systems the virulence ranking of several clinical strains is the same between mice and *Drosophila*.[143,144] *Drosophila* intestinal infection with *C. albicans* results in an extensive JNK-mediated death of gut cells and the expression of antimicrobial peptides in the fat body.[144] Moreover, *Candida* pathogens secrete aspartyl proteinases (SAPs), which are critical molecules that allow them to degrade barrier tissues by hydrolysing proteins such as collagen, fibronectin and keratin in order to obtain nutrition at the site of the infection.[87] In addition, the secretion of SAP4 and SAP6 from *Candida* is necessary for the activation of systemic Toll-dependent immunity.[144] Although Toll pathway controls fungal infection with both *C. albicans* and *C. glabrata*, the two species differ in their ability to activate protective melanization.[88]

Conclusion: Toll-dependent defense responses contribute to resistance although to a different extent against systemic *C. albicans* and *C. glabrata*. SAP proteases of *C. albicans* compromise the intestinal barrier function and contribute to pathology.

Cryptococcus neoformans

C. neoformans is another opportunistic fungal pathogen that can cause serious infections in immunocompromised patients, such as those with HIV/AIDS.[90] In addition, systemic *Cryptococcus* infection is associated with meningoencephalitis.[90] *Drosophila* S2 cells can be used in combination with RNA

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interference technology for identifying host defense factors and mechanisms, for example, the exploitation of host autophagy by *C. neoformans* to survive and disseminate upon infection.[90] Moreover, Toll pathway is critical for host defense when *C. neoformans* is introduced into the hemolymph of *Drosophila*, but Toll and Imd pathways are dispensable for host defense against intestinal infections.[91] Further studies showed that there are alternative, NFκB-independent, immune responses acting in the *Drosophila* intestine against many intestinal pathogens.[92]

Conclusion: Alternative routes of infection reveal the existence of intestinal defense pathways other than the Imd and Toll as critical for host defense, while host cell autophagy contributes to pathogenesis.

Cunninghamella bertholletiae and Rhizopus oryzae

C. berthollethiae and *R. oryzae* are filamentous fungi that cause invasive mucormycosis, and are associated with high rates of mortality, especially in immunocompromised patients, such as those with hematological malignancies.[93] In a *Drosophila* model of mucormycosis the virulence of *C. bertholletiae* isolates is affected by iron content the nutrient media in which fungi grow.[93] Similarly, corticosteroid drugs and deferoxamide that affect iron availability in the host also affect wild-type *Drosophila* infection with *C. bertholletiae*.[94] In addition, tarcolimus and posaconazole have been shown to have combinatorial efficacy against *R. oryzae* in flies and mice.[99]

Conclusion: *Drosophila* models of infection show that iron availability in the growth media and iron availability in the host affect the virulence of *C. bertholletiae* isolates. Tarcolimus and posaconazole show promise in combinatorial treatments against *R. oryzae*.

Viruses

Dengue virus (DENV)

Dengue virus can cause dengue fever which can develop into dengue hemorrhagic fever and dengue shock syndrome.[101,102] Infection of *Drosophila* S2 cells with four DENV serotypes (DENV1–4) induces an RNAi response. Knocking down the RNAi pathway results in 10- to 100-fold enhancement of replication of all strains tested.[101] In addition, a genome-wide RNA interference screen in *Drosophila* cells identified candidate host factors implicated in the propagation of DENV.[102] Eighty-two of these have human homologs, while 42 were previously known to affect virus replication in human cells.[102]

Conclusion: An RNAi response is triggered by DENV to control infection. Additional factors conserved between *Drosophila* and humans have been found to control infection and those could be further explored in mammals.

Epstein–Barr virus (EBV)

Epstein–Barr virus is associated with many different cancers,[105,106] but also with several autoimmune diseases.[107] Viral gene expression in *Drosophila* is used to identify host cell proteins that can modulate the functions of EBV immediate-early genes BRLF1 and BZLF1, which are essential for the EBV replication.[105,106] BRLF1 expression in fly tissues inhibits known tumor suppressor genes and as a consequence induces overproliferation.[106] Furthermore, many *Drosophila* genes with known human homologs are required for EBV induced cell proliferation.[106]

Conclusion: *Drosophila* is a model host system for identifying human genes, such as tumor suppressors that are targeted by BRLF1 and are relevant to EBV-mediated tumorigenesis.

Human immunodeficiency virus (HIV)

HIV is the cause of the acquired immunodeficiency syndrome (AIDS) and there is no vaccine against it. High HIV-1 replication in the host-cells is achieved by accessory proteins, including the viral protein U (Vpu).[112] Vpu expression in the *Drosophila* fat body results in the inhibition of Cactus degradation counteracting Toll pathway activation.[112] In addition, Vpu expression in the *Drosophila* wing primordia triggers apoptosis via JNK pathway signaling.[113]

Conclusion: Vpu inhibits Toll and induces JNK pathway, depending on the tissue in which it is expressed. Thus orthotopic expression of viral proteins in immune cells and barrier epithelia might be required for the study of responses elicited by Vpu.

Influenza A virus

Influenza is caused by negative-strand RNA viruses of the family *Orthomyxoviridae*. It is highly contagious and sometimes deadly.[114] Using a modified virus able to replicate in *Drosophila* cells 3 genes and their human homologs (ATP6 V0D1, COX6A1, and NXF1) were found to control viral replication.[114] In addition, expression of the influenza virus M2 gene in *Drosophila* led to the identification of V1V0 ATPase as a potentiator of M2-mediated aberrant cell development to the host cell.[115,116]

Conclusion: Adaptation of the virus for growth in *Drosophila* cells facilitates the identification of host genes that affect influenza A virus replication and aberrant host cell programming.

SARS coronavirus (SARS-CoV)

The severe acute respiratory syndrome–coronavirus (SARS-CoV) is the etiological agent of the 2003 atypical pneumonia outbreak.[120,121] The SARS-CoV3a locus encodes a 274 a.a. potassium channel protein, which is detected in the patient's cells.[122] This protein is usually localized on the cell surface of virus-infected cells.[122] *Drosophila* expressing the 3a protein is suitable for the investigation of its apoptotic function and genetic interaction with host factors, such as cytochrome c.[120,122] Likewise, expression of the SARS-CoV Membrane (M) structural protein in *Drosophila* induces apoptosis via the inhibition of the AKT pathway.[121]

Conclusion: *Drosophila* transgenic models of SARS-CoV indicate genetic interactions of the viral apoptotic proteins 3a and M with cytochrome c and the AKT pathway, respectively.

Simian vacuolating virus 40 (SV40)

Simian vacuolating virus 40 belongs to the family of DNA tumor viruses.[123] Such viruses induce host cell proliferation in order to promote their replication.[123] Expression of the viral oncogene tumor antigen ST in *Drosophila* tissues and mammalian cells leads to its interaction with PF2A and the induction of centromere duplication.[123]

Conclusion: The interaction of tumor antigen ST with PF2A and the concomitant centromere duplication may drive oncogenesis by SV40.

Sindbis virus (SINV)

Sindbis virus is a mosquito-borne alphavirus that can cause fever, arthralgia and rush in humans.[124] Natural resistance-associated macrophage protein (NRAMP), a host cell surface iron transporter with 12 transmembrane domains, is used by SINV to

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enter *Drosophila* cells in culture and in adult flies.[124] Consistently, SINV entry and infection of the mammalian cells is mediated by the NRAMP homolog, NRAMP2.[124]

Interestingly, arboviruses and food nutrients induce the ERK pathway, which in turn restricts viral infection in the *Drosophila* intestine. That is, SINV and vesicular stomatitis virus become infective upon genetic or pharmacological inhibition of the ERK pathway. Strikingly, vertebrate insulin, which activates ERK in the mosquito gut during a blood meal, restricts viral infection of the insect intestinal epithelium.[125]

Conclusion: NRAMP family proteins are used by the SINV α virus to enter *Drosophila* and mammalian cells. ERK pathway induction is pivotal for *Drosophila* and mosquito host intestinal defense.

Vesicular stomatitis virus (VSV)

Vesicular stomatitis virus, a member of rhabdovirus family,[189] is a highly promising agent for cancer treatment, since it selectively infects and kills cancer cells.[128] Recognition of this single stranded RNA virus by the *Drosophila* pattern recognition receptor Toll-7, similarly to mammalian TLR7,[130] results in the activation of antiviral autophagy, which is NFκB-independent.[129] Consistent with this, flies deficient for Toll-7 are more susceptible to VSV infection.[129] Other studies in both adult flies and *Drosophila* S2 cells also show that activation of autophagy in *Drosophila* decreases the replication of VSV.[190] Interestingly, the host cells recognize a preformed component of the virus and induce autophagy before the initiation of viral replication.[190]

Conclusion: Similarly to mammalian TLR7, *Drosophila* Toll-7 induces autophagy to suppress VSV infection in an NFκB-independent manner. Nevertheless, Toll-7 recognizes the viral capsid, as opposed to viral RNA recognition by the mammalian TLR7. Thus, similarly to Toll, Toll-7 pathway appears conserved in mammals, but only downstream of the receptor.

West Nile virus (WNV)

West Nile virus is emerging as a highly virulent human pathogen.[131,132] It belongs to neurotropic mosquito-borne flaviviruses[131,132] causing fever, meningitis and encephalitis. Similarly to VSV infection, WNV infection induces RNAi as a defense mechanism in *Drosophila*.[133] WNV infection of adult *Drosophila* also supports the idea of a triggered protective RNAi response upon infection.[133] Importantly, non-coding WNV and other flavivirus RNA can suppress the RNAi defense mechanism in mammalian and *Drosophila* cells.[131]

Conclusion: WNV can induce and suppress RNAi in *Drosophila* and mammals.

Shortcomings of *Drosophila* Models of Microbial Infections

Drosophila can be used to investigate many mechanisms underlying microbial infections in humans, but there are also limitations in its use due to the evolutionary distance between flies and mammals. Thus a gold standard in studying human pathogens in flies is to verify findings in mammalian models of infection. Focusing on conserved aspects of host immunity and physiology increases the chance that any mechanism of pathogenesis identified in *Drosophila* will have a direct impact in humans. For example, the *Drosophila* melanization, while clearly contributing to host defense, it does not appear conserved in mammals. Thus, caution should be taken when interpreting findings related to the fly melanization in terms of human infectious diseases.

Some aspects of wound healing and inflammation cannot be modeled in *Drosophila*, because particular cells and tissues found in mammals are missing from flies. For example, flies lack an adaptive immune response as we know it in humans, thus they are inappropriate for studying the impact of the known adaptive immunity on tissue repair and inflammation.[191] Also fibrosis and scarring cannot be easily investigated in *Drosophila* because there are no myofibroblasts and no connective tissues to induce fibrosis.[191] Additionally, flies lack structural orthologs of many mammalian effector molecules, including chemokines, which are crucial for cell communication and regulation of inflammation during infection.[138,191] Furthermore, due to the absence of lamina propria from the *Drosophila* intestine, which includes connective tissue, myofibroblasts, and immune cells, it is only possible to study regenerative inflammatory signals of the intestinal epithelium, trachea, and muscle.[2] For example, the local tissue-emanating signals in *Drosophila* that control regeneration of the intestinal epithelium upon damage or infection.[147]

Additional limitations may also be posed by the wrong choice of infection methods. For example, when *Drosophila* is injected directly into the hemolymph with various bacteria, flies can be killed even by bacterial strains that are considered nonpathogenic in mammals.[192] Thus, this technique might fail to distinguish between virulent and non-virulent bacteria,[192] in which case pathogenicity cannot be studied and alternative modes of infection should be tried. Accordingly, infection modes that mimic intestinal or wound infections might be more appropriate for highly virulent microbes, such as *P. aeruginosa*, *E. faecalis*, and *S. aureus* that initially exert their virulence locally on soft tissues. Importantly, while major differences in host survival to infection and bacterial load are mostly independent of the general genetic background, less extensive differences are not.[145] In the latter case isogenic fly strains should be compared or more than one wild-type and mutant fly strains for the same gene should be assessed.

Finally, while some mammalian viruses can be recognized by and can enter *Drosophila* cells, others need to be previously modified. Therefore in many cases only viral proteins can be assessed via transgene expression in fly tissues. While transgenic flies can produce valuable results they do not necessarily recapitulate the complexity of the whole virus and can only provide insights on specific aspects of the infection.

Conclusions

A better understanding of host–microbe interactions is critical for the development of successful treatments. *Drosophila* represents a very useful invertebrate model host for studying many human microbes. Similarly to humans, host–pathogen interactions in flies are far more complex than the induction of distinct immune responses directed against gram-negative or gram-positive bacteria and fungi or viruses. This is because microbial strain-specific

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virulence factors—identified in *Drosophila* and other hosts—and host factors control, not only innate immune responses, but also muscle homeostasis, intestinal regeneration, predisposition for cancer, and tolerance to infection. Prominent among those is the role of intestinal regeneration as a protective response induced by pathogens, such as *P. aeruginosa,* but also beneficial bacteria, such as *L. plantarum*. Interestingly, *V. cholerae* appears to have the ability to suppress regeneration and *S. marcescens* appears to benefit from the induction of regeneration. Clearly, future studies can shed more light into this exciting area of research.

Regarding the modeling of disease in flies, the route of infection plays a pivotal role in the interaction. Microbial injection into the hemolymph, for example, bypasses many of the host barrier defenses, and it might be appropriate to study systemic infections, but not highly virulent microbes able to bypass *Drosophila* barrier defenses. Finally, microbes that do not inflict disease in wild type or even in immunocompromised flies can still be studied if their virulence factors are genetically expressed preferentially orthotopically in fly tissues homologous to those relevant to human pathophysiology.

Much of the knowledge gained from *Drosophila* studies of human microbes is and will continue to be important for biomedical research because most infection models strive to recapitulate conserved aspects of human disease. Despite the existence of rough guidelines, there is no strict formula of success in modeling human disease in flies. Thus, validation of any new findings conventionally necessitates the use of mammalian models of infectious disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Drosophila melanogaster: a first step and a stepping-stone to anti-infectives

Ilias Tzelepis[1], Stefania-Elisavet Kapsetaki[1], Stavria Panayidou[2] and Yiorgos Apidianakis[2]

Following an expansion in the antibiotic drug discovery in the previous century, we now face a bottleneck in the production of new anti-infective drugs. Traditionally, chemical libraries are screened either using *in vitro* culture systems or *in silico* to identify and chemically modify small molecules with antimicrobial properties. Nevertheless, almost all compounds passing through *in vitro* screening fail to pass preclinical trials. Drug screening in *Drosophila* offers to fill the gap between *in vitro* and mammalian model host testing by eliminating compounds that are toxic or have reduced bioavailability and by identifying others that may boost innate host defence or selectively reduce microbial virulence in a whole-organism setting. Such alternative screening methods in *Drosophila*, while low-throughput, may reduce the cost and increase the success rate of preclinical trials.

Addresses
[1] Department of Biology, University of Crete, Heraklion, Greece
[2] Department of Biological Sciences, University of Cyprus, Nicosia, Cyprus

Corresponding author: Apidianakis, Yiorgos (apidiana@ucy.ac.cy)

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Chemicals related to this manuscript

Isoniazid, pyrazinamide, voriconazole, terbinafine, tarcolimus, posaconazone, 2-aminoacetophenone, deferasirox, methyl paraben and propionic acid.

Introduction

A common countermeasure to the ever-growing antibiotic drug resistance is the production of new effective drugs. Nevertheless, the rate of production of new antibiotics is steadily declining [1]. One reason for this might be the chemical screening methods that rely solely on *in vitro* culture systems. Traditionally, drug research is moving from *in vitro* small molecule screens to preclinical assessment in mammalian hosts. There are two problems with this approach: first, tests in mammals are costly and can usually be restricted to a few compounds at a time, and second, *in vitro* assays are inappropriate to capture the complexity of an infected host [2]. Live hosts are preferable because they enable drug toxicity and bioavailability assessment at the organismal level [3] and [4]. In addition, drugs that might interfere with the host microenvironment or microbial virulence *per se* can only be assessed upon the interaction of microbes with a host. Thus, quality anti-infective drug assessment in simple model hosts might be a more effective way to identify new drug leads. In this review, we aim to examine the suitability of *Drosophila melanogaster* as a model organism for anti-infective drug assessment due to its high degree of molecular, cellular and physiological conservation with humans, which allows the modelling of infections that recapitulate aspects of human disease [5•,6]. In this respect, *Drosophila* might fill the gap between *in vitro* screens and preclinical trials or be used directly, instead of *in vitro* screens.

Advantages of *Drosophila* in terms of laboratory use

Drosophila has a short life cycle of ~10 days from egg to sexually mature adult as compared to the ~2.5 months of mice (Table 1). Large numbers of flies can be propagated quickly, since tens of females can produce hundreds of offspring within two weeks. The offspring become sexually mature very early in their adult life, enabling the life cycle to continue [2]. Due to its small size of 2 mm in length thousands of flies can be contained in a space that would normally fit less than 10 mice. In addition, fly food is usually made of grocery store ingredients such as cornmeal, yeast and sucrose, thus the cost of maintenance is quite low. Moreover, there are no ethical concerns or regulated protocols for its use in biomedical research.

As an advantage over *Caenorhabditis elegans*, a popular invertebrate model host, drugs can not only be mixed in the fly food but also administered by injection (Table 1). Precise doses of 2–200 nl of drug solutions can customarily be injected in each fly [7] and less than 200 μl on a paper disc suffice to feed 20 flies for 24 hours [8•]. Hence, only small quantities of drugs are required during experiments; yet another reason why drug tests in flies are not expensive. In addition, *Drosophila* can be used for toxicological studies because the relative toxicity of chemicals in flies correlates well with that in mammals [9]. Finally, *Drosophila* infection and inflammation can easily

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Table 1

Comparison of model organisms most commonly used in drug discovery

		C. elegans	*Drosophila*	Mouse
Practical aspects	Embryogenesis and sexual maturation	3 days	~10 days	~2.5 months
	Size	1 mm	2 mm	10 cm
	Cost	Low	Low	Medium
Similarity to humans	Number of genes	21,187	15,867	34,293
	Disease homologs	~65%	~75%	~95%
	Physiology	Low	Medium	High
	Innate immunity	Low	Medium	High
Genetic tools	Whole-genome RNAi	Yes	Yes	No
	Tissue/time specific RNAi	No	Yes	No
	Gene knockouts	~50%	~50%	~10%
	Transgenesis	Easy	Easy	Laborious
Drug testing	Drug delivery	Feeding	Feeding-injection	Feeding-injection
	Drug quantity	μl	μl–nl	ml–μl
	Throughput	High	Low	Very low

be studied in relation to aging overcoming the barrier of long experimental time [10]. This is because *Drosophila* maximum life span ranges between 60 and 90 days, with 1 day of the fly roughly corresponding to 1 year of humans. That is, flies exhibit aging effects as early as 20 days post the onset of adulthood.

Advantages of *Drosophila* genetics

Drosophila has a long history as a model organism for genetics and a significant similarity with humans in terms of gene homologs. It has functional homologs for 75% of human disease related genes [11], more than any other invertebrate model host studied today (Table 1 and [12]). Its genome is fully sequenced and is one of the best-annotated among eukaryotes. Thus, many technologies have been developed and techniques are easily and commonly used, such as transgenesis, RNA interference (RNAi) technology and gene microarrays. Double-stranded RNAs have been synthesized for almost all genes and the tools are commercially available for the conditional inactivation of essentially any gene of interest *in vivo* or in cell culture [13]. For example, *Drosophila* cells have been used in genome-wide RNAi screens to rapidly identify genes required for replication of influenza and dengue viruses [14,15]. Furthermore, there are large collections of mutants and transgenic *Drosophila* stocks maintained at Bloomington and other stock centers around the world (http://flybase.org). Moreover, the *Drosophila* genome contains fewer genes than humans, and consequently, presents less overall genetic redundancy. This allows for an easier target identification, although multiple or modified drugs might be needed in mammals to affect the multiple gene variants. Finally, a variety of genetic tools and markers are available today in order to study the role of microbial pathogenicity tissue-specifically using the GAL4/UAS system [2]. This is an advantage over other model hosts, because expression of any *Drosophila* gene can be controlled time and tissue-specifically (Table 1). For example, tissue-specific and temporal RNAi allowed the identification of the JAK/STAT signalling pathway as a regulator of the intestinal immune response and regeneration in the fruit fly [16]. In addition, intestinal damage and regeneration can be studied by flip-out clones of cells emanating from intestinal stem cells [17,18], as well as mitotic clones using either the β-galactosidase marker or the ''Mosaic Analysis with a Repressible Cell Marker'' method [2].

Drosophila physiology and the immune system — conservation and significance for mammalian research

Several organs and specific cells fundamental to the immune response are highly conserved between flies and mammals. This is the most significant advantage over all other invertebrate model hosts studied today (Table 1). Flies have a defined brain that interacts with other organs, for example, the fat body and the intestine *via* cytokines and insulin peptides, respectively [19,20]. The fat body is the equivalent of the mammalian liver, an innate immunity and a metabolic organ [19,20]. The fly intestine bears many similarities with that of mammals in terms of cellular and molecular biology and epithelial architecture [2]. Plasmatocytes are the macrophage-like cells of *Drosophila* that detect and phagocytose microbes and secrete cytokines and antimicrobial peptides [21]. The muscle cells of the *Drosophila* flight muscle, heart and intestine are stratified or smooth similarly to those of humans and share a role in host response to infection [2,22]. The *Drosophila* trachea is an air-transporting organ with similarities to the human vasculature [23]. Finally, the nephrocytes and the malpighian tubules are kidney-like cells with a role in host defence [24,25].

The *Drosophila* epithelia that are attached to the cuticle, as well as those of the intestine and the trachea are physical barriers to pathogen invasion and the first to respond to external microbes. Should microbes invade

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these epithelia other local tissues, such as the *Drosophila* flight muscle, respond to wound infection eliciting a localized host defence response orchestrated by the highly conserved JNK pathway [22]. Importantly, muscle responses to wound infection appear to be conserved in mice and in humans [22,26]. On the other hand, when bacteria enter and damage the intestine, they induce enterocyte regeneration, which serves as a defence response to protect the host [17]. Numerous conserved signalling pathways are involved in intestinal regeneration upon infection, including the Wnt/Wg, Notch, Hippo, JNK, INSR/InR, K-Ras/Ras1, JAK-STAT and the NF-κB pathways [27].

In case microbes pass through intestinal or other barrier epithelia, additional mechanisms of protection take place. These include phagocytosis by the plasmatocytes, which are analogous to the mammalian macrophages, and the production of antimicrobial peptides by the fat body [21]. Many bacteria and fungi induce the Toll and/or the immune deficiency (Imd) pathways, which are the two highly conserved NF-κB pathways of the systemic *Drosophila* immune response [27].

Viral infections elicit systemic immune responses *via* the universally conserved RNAi mechanism. The *Drosophila* small interfering RNA pathway is activated by double-stranded viral RNA or DNA [28]. Moreover, DExD/H box helicases, cell autophagy as well as the conserved JAK/STAT, Imd/TNF and/or the Toll/TLR innate immune cascades play a crucial role in responding to viral RNA in flies and mammals [6].

Human related microbes studied in *Drosophila*

Many human bacterial pathogens have been studied in *Drosophila* including the Gram-positive bacteria *Enterococcus faecalis*, *Staphylococcus aureus*, *Steptococcus pneumoniae*, *Bacillus cereus* and *Listeria monocytogenes*, and the Gram-negative bacteria *Vibrio cholerae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Chlamydia spp.*, *Burkholderia cepacia*, *Yersinia pseudotuberculosis*, *Francisella tularensis*, *Legionella pneumophila and Mycobacterium marinum* [7]. Of those, *P. aeruginosa* and *M. marinum* may suppress the innate immune response as part of their virulence repertoire [22,29]. Interestingly, the antibiotics rifampicin, dinitrobenzamide, amikacin and isoniazid show good bioavailability, because when fed to the flies they alleviate systemic *M. marinum* infection. Of special note, the success of the antituberculosis drugs isoniazid and pyrazinamide against the tuberculosis model microbe *M. marinum* is facilitated by a boost in host cell autophagy in flies and mammals [30••]. These data suggest that not only direct antibacterial efficacy but also innate immune induction share similarities between flies and mammals and can be exploited for pharmacological assessments in flies.

Intestinal *P. aeruginosa* induces damage and apoptosis of midgut enterocytes in *Drosophila*, which in turn induces intestinal stem cell proliferation, a process that is however reversible upon bacteria clearance by the common food preservatives methyl paraben and propionic acid [17]. Strikingly, K-Ras/Ras1 oncogene expressing *Drosophila* hindgut cells induce tumors and delaminate through the basal side of the epithelium upon *P. aeruginosa* infection, which is an additional process that can be inhibited by eliminating infection using food preservatives [31•,32]. Furthermore, 2-aminoacetophenone, a small chemical produced by *P. aeruginosa*, has been shown to reduce *P. aeruginosa* virulence in *Drosophila* and mice [8•]. Finally, researchers have exploited phages as anti-infectives against *P. aeruginosa* using *Drosophila*. Fruit flies infected with *P. aeruginosa* can be treated with bacteriophages MPK1, MPK6 by feeding [33,34•]. Such findings encourage future assessment of food preservatives and natural or biological products, including bacterial metabolites and bacteriophages, as anti-infectives.

Apart from bacteria, human fungal pathogens can also inflict disease in flies. *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus hyphae*, *Cryptococcus neoformans*, *Cunninghamella berthollethiae*, *Scedosporium* spp. and *Fusarium* spp. have been studied in flies [13,35]. Of those, the zygomycete *C. berthollethiae* has been meticulously studied in combination with chemical modifiers of iron in *Drosophila*. Enhancers of zygomycetes virulence traditionally used in humans, such as corticosteroids, increase iron supply, and iron availability through treatment with deferoxamine dramatically increases pathogenicity by zygomycetes. Accordingly, iron starvation induced by treatment with the iron chelator deferasirox significantly protects infected flies [36]. Another common antifungal, voriconazole is potent against *F. moniliforme* and *S. apiopermum* infection in flies [37]. Moreover, combinatorial drug assessment assays in *Drosophila* reveal a synergism between voriconazole and terbinafine against *Aspergillus fumigatus*, similar to that seen in mammals [38]. Recently, another synergy was shown between tarcolimus and posaconazone in flies and mice against *Ryzopus oryzae* [39•]. Because all of the aforementioned treatments were administered by feeding in flies, while infections were either superficial or systemic, many antifungal drugs appropriate for humans may have good bioavailability and efficacy in flies.

Human related viruses that have been studied in flies include, Dengue virus, Epstein-Barr virus, Hepatitis B virus, Human cytomegalovirus, HIV-1, Influenza A virus, SARS coronavirus, Simian valuolating virus 40, Vaccinia virus, Sindbis virus, Vesicular Stomatitis virus and West Nile virus [6]. The last three of those have also been studied in adult flies, thus allowing the assessment of treatments against them in a whole organism setting. Pertinent to the identification of gene target against these

viruses, *Drosophila* NRAMP and its human homologue NRAMP2 have been identified as necessary for the entry of Sindbis virus into the host cells [40••]. In addition, *Drosophila* Toll-7 has been identified similar to its mammalian ortholog TLR-7 as important for host defence to infection against Vesicular Stomatitis virus *via* the induction of cell autophagy [41]. Finally, West Nile virus 3'-untranslated region-derived RNA molecule, known as subgenomic flavivirus RNA, suppresses the siRNA-induced and miRNA-induced RNAi pathways in both mammalian and insect cells [42], indicating that RNAi-based therapies might be a goal for the near future against insect-borne flaviviruses.

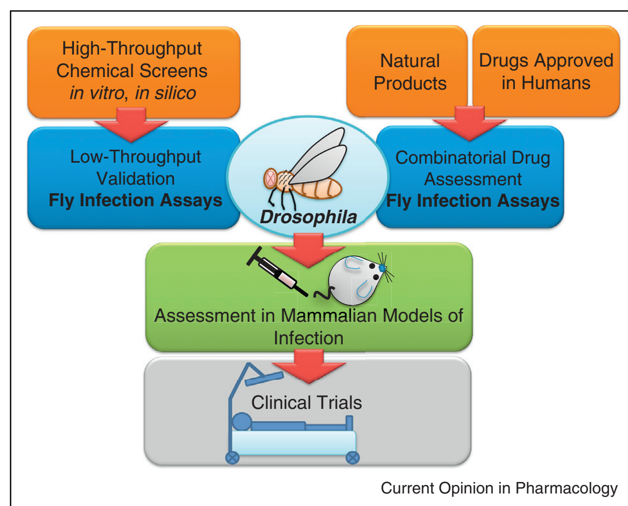
Obstacles and disadvantages of the model

Despite the numerous advantages of *D. melanogaster* as a model organism for the study of anti-infectives, there are also several shortcomings. That flies are infected and maintained at a temperature of 25–29°C can be a problem for the study of pathogens and virulence factors that require the mammalian body temperature, that is, 37°C [13]. Also, its inability to simulate human intestinal anaerobic microflora can be a disadvantage. While micro-aerophilic and aerotolerant bacteria might be used to infect flies, the presence of oxygen in the fly intestine prohibits fly infections with strict anaerobes, which are plentiful in the human gut [43]. Nevertheless, as with any microbe that is difficult to establish an infection with, specific virulence factors can be expressed or administered to flies to study their virulence. Moreover, pharmacokinetic analyses are still problematic in insects as there is not a precise method to measure the levels of administered drug tissue-specifically and insect xenobiotic metabolism might be very different from that of mammals. Furthermore, as opposed to mammals, *Drosophila* lacks an adaptive immune system and specialized immune response cells, such as dendritic cells (DC), B and T lymphocytes, which are responsible for immunological specificity and memory [44]. In addition, despite the significant conservation of the core of *Drosophila* signalling pathways, some of them might be activated differently between flies and mammals. For example, the mammalian Toll/TLR pathway that is directly activated by microbially associated molecular patterns, while the *Drosophila* Toll is activated indirectly through a cascade of proteases [45] and the mammalian TLR-7 that is localized in intracellular membranes versus the plasma membrane-localized *Drosophila* Toll-7 [41]. Finally, high-throughput screening for anti-infectives has not been developed in *Drosophila* and this is its major drawback as compared to other invertebrate hosts (Table 1).

Concluding remarks and Future perspectives

In recent years, the conventional methods used in most pharmacological studies for the discovery of new therapeutic drugs are based either on screening of small molecule libraries for the capacity to induce a specific phenotype *in vitro* or *in silico* [46,47]. However, the efficacy of these methods is very low, because they lack the complex and dynamic host–pathogen interactions, which occur *in vivo*. Consequently, the use of mammalian hosts in such studies is needed and seems to be very widespread and prevalent nowadays. Even so, using a conventional animal model for this purpose can be time-consuming, laborious and expensive, not to mention the ethical concerns. Exploiting alternative strategies, *D. melanogaster* is a very promising and useful host, which may cover this gap between the computational or cellular testing studies and the tests in mammals (Figure 1). While low-throughput drug assessment in *Drosophila* has been proven meaningful, large-scale assessments might also be possible on the basis of protocols used for the identification of molecules that modify disease progression in Fragile X syndrome though a screen of 2000 compounds in *Fmr1*-mutant flies [48] and a screen of 1280 small molecules that identified reserpine as a sleep regulator [49]. In addition, the fly can be used to assess drugs already approved for human use (Figure 1). Indeed, the efficacy of a number of licensed anti-infective agents has been evaluated in *Drosophila*, demonstrating a significant correlation in drug efficacy between flies and mammals. Therefore, the use of *Drosophila* for anti-infective drug discovery may be a promising auxiliary tool for preclinical research.

Figure 1



Drosophila can be used either to validate candidate drugs or in combinatorial drug assessment assays to identify synergistic drug combinations. Flies have significant similarities with humans enabling a facile and cost effective assessment of anti-infective drugs during the interaction of microbes with a host. Hits selected from *in vitro* or *in silico* chemical screens can be further screened in *Drosophila* survival or microbial colonization assays to select drug candidates that will have a higher success rate in preclinical trials. In addition, natural products, for example, microbial secondary metabolites and drugs approved in humans can be tested for the fist time combinatorially in flies to identify synergistic effects between two or more chemicals.

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Review

Regenerative Inflammation: Lessons from *Drosophila* Intestinal Epithelium in Health and Disease

Stavria Panayidou and Yiorgos Apidianakis *

Department of Biological Sciences, University of Cyprus, Nicosia 1678, Cyprus; E-Mails: spanay06@ucy.ac.cy (S.P.); apidiana@ucy.ac.cy (Y.A.)

* Author to whom correspondence should be addressed; E-mail: apidiana@ucy.ac.cy; Tel.: +357-22-893-767; Fax: +357-22-892-881.

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Abstract: Intestinal inflammation is widely recognized as a pivotal player in health and disease. Defined cytologically as the infiltration of leukocytes in the lamina propria layer of the intestine, it can damage the epithelium and, on a chronic basis, induce inflammatory bowel disease and potentially cancer. The current view thus dictates that blood cell infiltration is the instigator of intestinal inflammation and tumor-promoting inflammation. This is based partially on work in humans and mice showing that intestinal damage during microbially mediated inflammation activates phagocytic cells and lymphocytes that secrete inflammatory signals promoting tissue damage and tumorigenesis. Nevertheless, extensive parallel work in the *Drosophila* midgut shows that intestinal epithelium damage induces inflammatory signals and growth factors acting mainly in a paracrine manner to induce intestinal stem cell proliferation and tumor formation when genetically predisposed. This is accomplished without any apparent need to involve *Drosophila* hemocytes. Therefore, recent work on *Drosophila* host defense to infection by expanding its main focus on systemic immunity signaling pathways to include the study of organ homeostasis in health and disease shapes a new notion that epithelially emanating cytokines and growth factors can directly act on the intestinal stem cell niche to promote "regenerative inflammation" and potentially cancer.

Keywords: *Drosophila*; innate immunity; inflammation; cancer; regeneration; intestine

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1. Introduction

Inflammation is the physiologic response to tissue injury or infection. In its acute form, it is vital for tissue repair, homeostasis reinstatement, and organism survival. The medical hallmarks of inflammation are: pain (dolor); redness (rubor); heat (calor), which refers to an increase in temperature due to vascular dilatation and delivery of warm blood to the area of the tissue damage; swelling (tumor), *i.e.*, fluid accumulation in the extravascular space and the migration of the inflammatory cells into the area; and, loss of function (functio laesa). Inflammation can become systemic, thus affecting the whole body rather than only one part of it. At the cellular level, inflammation involves the activation of tissue-specific (e.g., mast cells for the intestinal epithelium) and non-tissue-specific (e.g., macrophages, neutrophils, dendritic cells, T-cells, B-cells) cell types of the immune system [1]. Macrophages and neutrophils are the first line of immunity against invading pathogens [1]. However, if tissue homeostasis is perturbed, these cells release soluble factors, including cytokines and chemokines, in order to attract additional leukocytes into the site of damage [1]. Nevertheless, in chronic inflammation, the chronically perturbed tissue homeostasis creates a condition of a "wound that does not heal" that predisposes for cancer development [2,3].

In this review, we focus on intestinal inflammation and the conditions that may contribute to cancer. The maintenance of intestinal homeostasis requires a balance between the intestinal epithelial cells, the immune system, and the gut microbiota [4]. *Drosophila melanogaster* is a simple model where the mechanisms underlying processes like intestinal stem cell proliferation, differentiation and maintenance can be easily studied due to the evolutionarily conserved signaling pathways between *Drosophila* and mammals [5]. Many of these pathways are also activated in the *Drosophila* midgut upon bacterial infection and intestinal epithelium damage, and they are involved in the regeneration of the midgut epithelium. We review the *Drosophila* and the mammalian responses to stress or infection to conclude that epithelially emanating regenerative inflammatory signals similar to those derived from mammalian inflammatory epithelial cells or tumor cells *per se* may directly contribute to cancer initiation, maintenance and progression.

2. Early Lessons from *Drosophila* Systemic Immune Response

2.1. Drosophila Systemic Immune Response

During the past 20 years, flies have become an attractive model for studying innate immunity. Numerous studies show that *Drosophila* responds to bacteria, fungi, and viruses *via* the activation of highly conserved pathways e.g., the Imd, Toll, JNK and JAK/STAT pathways, leading to the systemic expression and release of antimicrobial peptides (AMPs) and other factors by the fat body and the hemocytes into the hemolymph [6]. The expression of AMPs is regulated by two critical NF-κB pathways, which are activated by bacteria and fungi [6] (Figure 1). The Toll pathway is induced by many bacterial and fungal species, which are recognized by secreted factors, such as GNBP1, PGRP-SA, PGRP-SD and GNBP 3, and which are all able to mediate the proteolytic cleavage and maturation of the Toll receptor-ligand Spätzle (Spz) [7,8]. Toll activation by ligand binding is followed by the recruitment of a receptor–adaptor complex consisting of three death-domain proteins: MyD88, Tube and Pelle [8,9] (Figure 1). Pelle phosphorylates the IκB-like protein Cactus, leading to its

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dissociation from the NF-κB-like transcription factor(s) Dorsal and/or Dif, thus allowing them to translocate into the nucleus and activate transcription of AMP genes [8,9]. The second NF-κB pathway that regulates AMP expression in *Drosophila* is immune deficiency (Imd), which is induced by many Gram-negative bacteria through the transmembrane PGRP-LC and the intracellular PGRP-LE peptidoglycan recognition proteins (PGRPs) [7,8] (Figure 1). Interestingly, the Imd signaling pathway involves a Tak1/Tab2 complex, which activates the JNK pathway allowing the nuclear translocation of AP-1 and the IKK complex that regulates the activation of the NF-κB-like protein Relish [7,9].

Furthermore, *Drosophila* infection with bacteria or viruses results in the activation of the JAK/STAT pathway, which is another evolutionarily conserved pathway with multiple roles in development and immunity (Figure 2). Induction of the JAK/STAT pathway following septic injury is mediated by the hemocyte-secreted cytokine Upd3, which is the ligand of the receptor Domeless (Dome) [10].

2.2. Mammalian Systemic Immune Response and Parallels with Drosophila

In contrast to the indirect recognition mechanism of the fly Toll, the mammalian Toll-like receptors (TLRs), are activated *via* direct binding to pathogen-associated molecules [9]. An oligomer complex similar to *Drosophila* MyD88, Tube and Pelle is utilized during mammalian TLR signaling: IRAK4 and IRAK1 are the mammalian orthologs of the *Drosophila* Tube and Pelle, respectively, while mammalian MyD88 recruits IRAKs and TRAF6 for the activation of the TAK1/TAB complex [7,11]. The downstream signaling is divided into two branches: the first branch emanating from TAK1/TAB stimulates the IKK complex for NF-κB activation and its translocation to the nucleus; and the second branch activates the MAPKKs pathways ERK, JNK and p38. JNK activation induces phosphorylation and nuclear translocation of the transcription factor AP-1 [7,9]. Thus the mammalian TLR pathway has high homology also with the *Drosophila* Imd pathway, downstream of the TAK1/TAB complex [7] (Figure 1). AP-1 comprises a group of sequence-specific transcription factors, which are conventional substrates for JNK and p38 [12]. JNK and p38 belong to the family of mitogen-activated protein kinases (MAPKs), which include the extracellular-signal-regulated kinases (ERKs) and ERK5 subfamilies [12,13]. Moreover, JNK and p38 are mainly activated by pro-inflammatory cytokines in response to stress, while ERK is induced by growth-promoting mitogenic stimuli [13]. TLRs mainly recognize pathogen-associated molecular patterns (PAMPs) in the extracellular environment [14]. However, there is another family of mammalian receptors, known as NOD-like receptors (NLRs), that sense a variety of ligands within the cytoplasm [15]. Similarly to *Drosophila* PGRPs, NOD1 and NOD2 sense peptidoglycan (PGN) fragments (iE-DAP and MDP, respectively) and activate RIP2, which is a serine/threonine kinase homolog of the *Drosophila* Imd [16] (Figure 1). Signaling through RIP2 leads to the activation of NF-κB and the production of inflammatory cytokines, while NOD2 signaling pathway additionally leads to the activation of MAPKs [16]. NF-κB can also be activated by tumor necrosis factor-α (TNFα) signaling [17]. Interestingly, NF-κB activation is regulated by two factors, the *cellular inhibitor of apoptosis 1* and *2* (cIAP1 and cIAP2) [17]. These factors are homologs of the *Drosophila* Imd pathway factor IAP2 (Figure 1). IAP2 is required for the sustained antimicrobial peptide gene expression in the *Drosophila* S2 cells [18].

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Mammalian cytokines that belong in the type I interferon (IFN) family induce innate immunity responses against viral infections through STAT1 kinase [19] (Figure 2). On the other hand, the mammalian STAT3 is a main regulator of the differentiation and development of adaptive immunity cells [20,21]. In addition, it mediates the transition from initial innate immune response to infection to a sustained adaptive immune response and has critical roles in inflammation and cancer [22]. It can be activated by IL-6, which is homologous to the *Drosophila* Upd cytokines (Figure 2). IL-6 usually binds to its receptor IL-6R and activates of the signal transducer gp130, *via* the threonine kinase JAK, which subsequently activates the transcription factor STAT3, inducing its nuclear translocation and DNA binding [20]. Moreover, the mammalian IL-6 receptor family members and the gp130 are homologs of the *Drosophila* receptor Domeless and the Eye Transformer (ET), respectively, although the latter is a negative regulator of *Drosophila* JAK/STAT pathway [23]. Pertinent to the negative regulation of the pathway, the *Drosophila* SOCS36E and PIAS have a highly conserved role similar to mammalian SOCS3 and PIAS3, respectively, in inhibiting signal transduction [24–27] (Figure 2).

3. Epithelial Immune Responses of Flies and Mammals

3.1. Drosophila Epithelial Immune Responses

In *Drosophila*, ROS and AMPs help the host to fight infection. However, ROS can also damage host cells. To protect the enterocytes from excessive ROS, *immune-regulated catalase* (IRC), is expressed as a response to oxidative stress during gastrointestinal microbial infection [28,29]. The *Drosophila* intestinal immune response depends on whether the invading bacteria are resistant to oxidative stress or not. In the case of ROS-sensitive bacteria, ROS production by Duox fights infection, while detoxification of ROS by IRC protects the host [28,30]. ROS-resistant bacteria may persist in the *Drosophila* intestine and activate the Imd/Relish pathway and subsequent AMP production for the neutralization of bacteria sensitive to AMPs [30]. However, JAK/STAT signaling can also contribute to AMP production [31,90].

Recently another ROS protection gene was shown to protect the host during intestinal bacterial infection in *Drosophila*. The JNK/FOXO pathway regulates the expression of the antioxidant enzyme Peroxiredoxin V (dPrxV) to protect intestinal epithelial cells from oxidative damage, as, for instance, dPrxV mutants exhibit increased lethality during bacterial infection [32]. However, the role of JNK is controversial: Upon aging, oxidative stress leads to abnormal proliferation and differentiation of intestinal stem cells *via* JNK signaling [33], but systemic JNK signaling results in less oxidative damage and lifespan extension [34]. These findings indicate that, on the one hand, JNK signaling induces expression of cytoprotective genes in response to increased stress and oxidative challenge, and, on the other hand, it mediates aberrant stem cell proliferation in the aged enterocytes of *Drosophila* [33,34]. FOXO, a target of JNK, is a transcriptional factor that can influence many biological processes including stress resistance. Under normal conditions it is cytoplasmic in intestinal epithelial cells, while upon intestinal infection it accumulates in the nucleus [32]. Importantly, the expression of dPrxV depends also on the expression of Duox, which generates bactericidal reactive oxygen species (ROS) upon infection [32]. Duox in *Drosophila* is induced by non-peptidoglycan (non-PGN) ligands, which are recognized by G-protein-coupled-receptors (GPCR) and induce the Gaq-PLCβ-IP3-Ca^{2+}

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pathway (Figure 1), resulting in ROS production in order to maintain balanced gut–microbe interactions [35]. In the absence of infection, the GPCR pathway suppresses the Imd-dependent Duox expression even in the presence of PGN [35]. However, when bacterial infection takes place, PGN induces Duox production *via* PGRP-LC–IMD–MEKK1–p38 signaling and non-PGN stimuli activate MEKK1 through GPCR-Gaq-PLCβ-MEKK1 signaling, resulting in maximal ROS production [35] (Figure 1).

Figure 1. Component conservation among the *Drosophila* and mammalian innate immunity NF-κB pathways. Homologs of the *Drosophila* Toll, Imd, GPCR and Eiger pathways and the mammalian TLR, NLR, TNF and GPCR pathways are marked with circles of the same color at the left of each component. Notice the striking homology of components between species, though some homologs are positioned in different pathways. The subcellular localization of homologous proteins is also conserved. AMPs: Antimicrobial Peptides; Duox: Dual oxidase; GPCRs: G-Protein-Coupled Receptors; NLRs: NOD-like Receptors; Nox: NADPH oxidase; PGN: peptidoglycan; PAMPs: Pathogen-Associated Molecular Patterns; PGRPs: Peptidoglycan Recognition Proteins; ROS: Reactive Oxygen Species; TLR: Toll-like Receptors; TNF: Tumor Necrosis Factor.

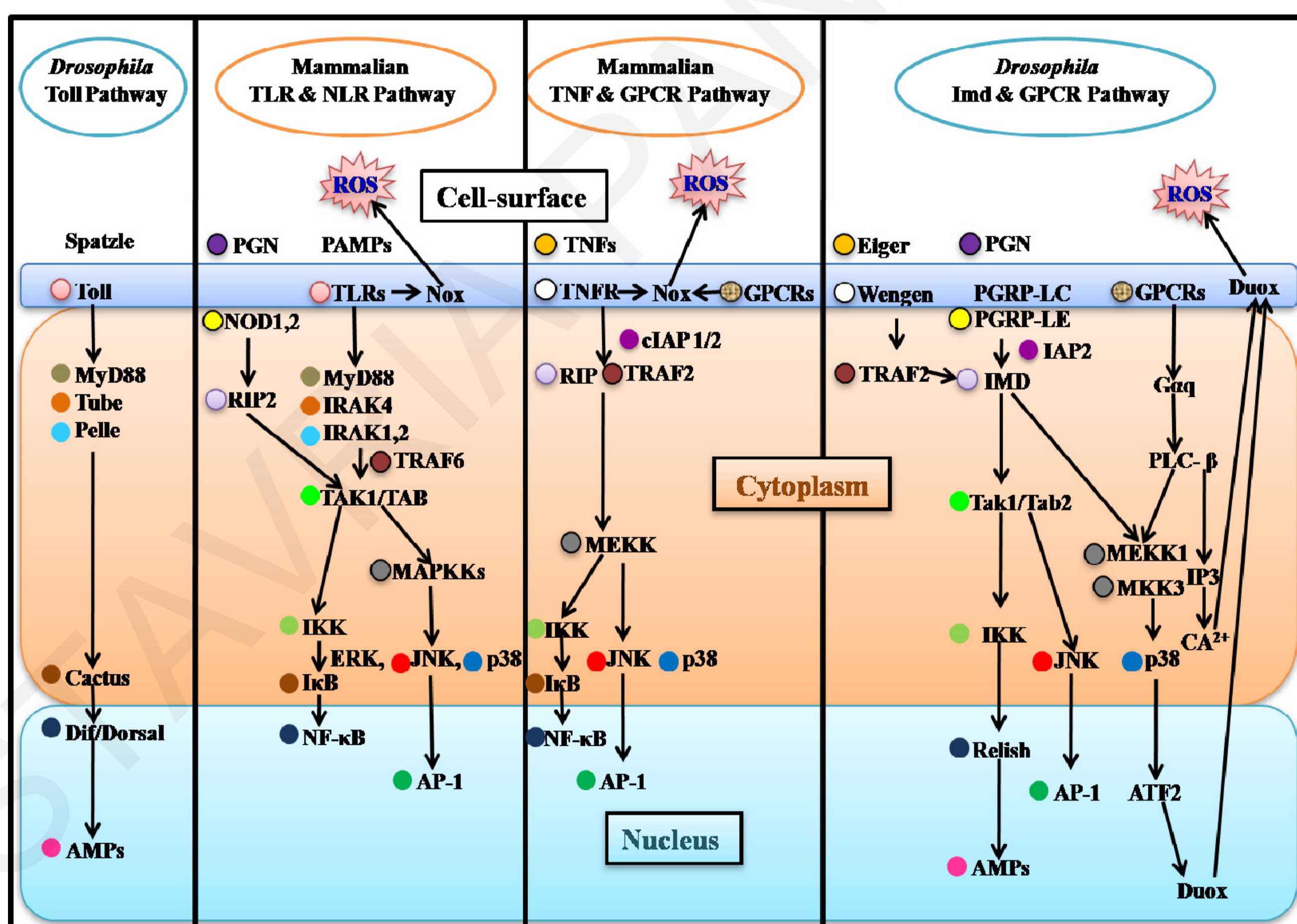
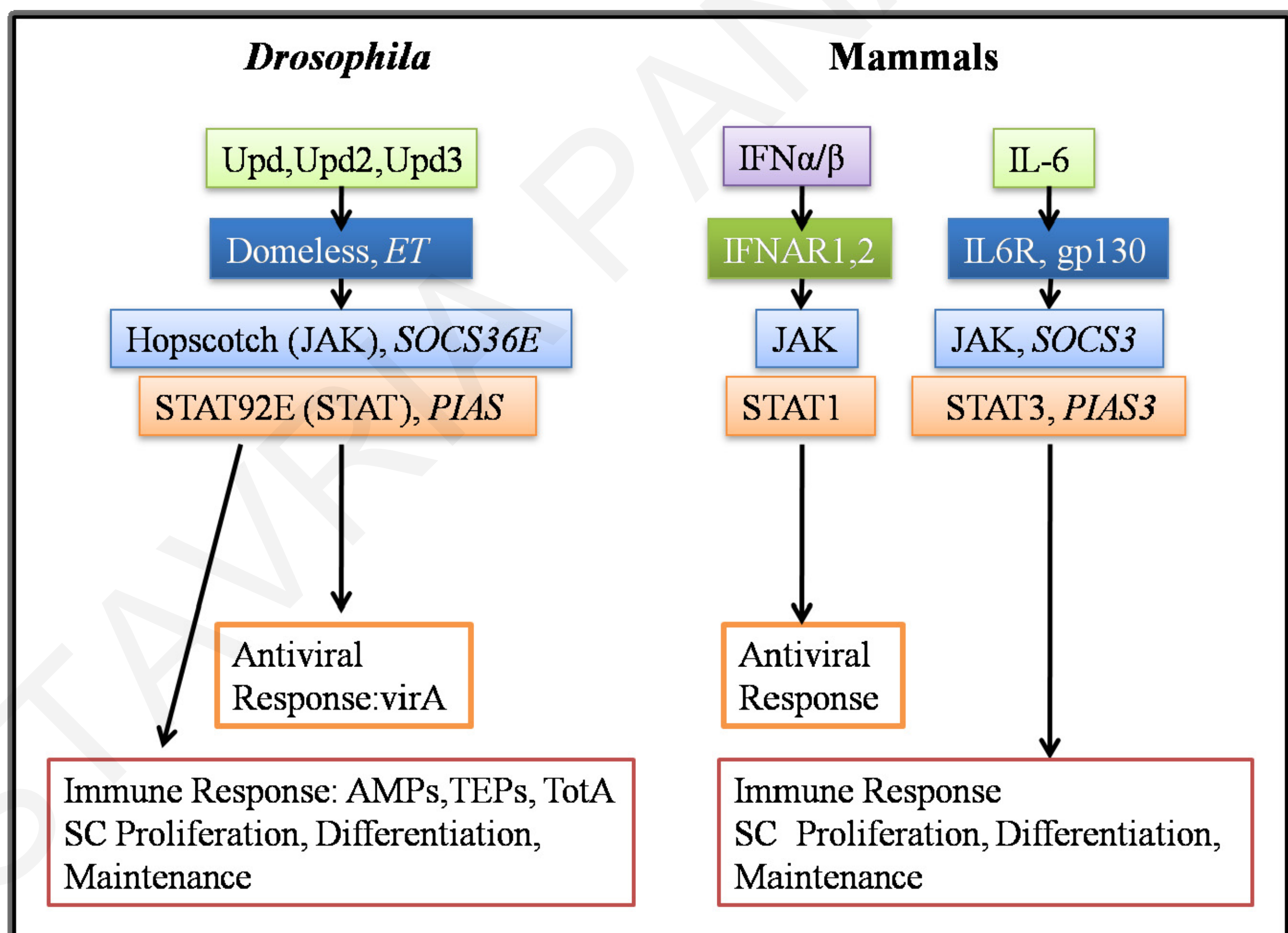


Figure 2. Conservation between the *Drosophila* and the mammalian JAK/STAT pathway and their commonalities in inducing systemic and localized immune response and tissue growth. Activation of the *Drosophila* JAK/STAT pathway by the Upd (Upd, Upd2 and Upd3) cytokines has critical roles in many developmental processes, as well as in immune responses. The JAK/STAT pathways in mammals can be activated by different ligands (e.g., IL-6 and IFNs) that induce distinct signaling cascades. IL-6 binding to its receptor induces innate immunity and tissue growth and maintenance, similarly to the activation of the *Drosophila* JAK/STAT signaling by the Upds. JAK/STAT activation by IFNs promotes antiviral activities. Rectangles of the same colors indicate the homology between the components of *Drosophila* and mammalian JAK/STAT pathway components. Components in italics *i.e.*, the *Eye Transformer (ET)*, *PIAS*, *PIAS3*, *SOCS36E* and *SOCS3* are negative regulators acting at the point of the pathway where they are placed. AMPs: Antimicrobial Peptides; ET: Eye Transformer; JAK: Janus Kinase; IFNAR: Interferon-α/β Receptor; PIAS: Protein Inhibitor of Activated STAT; SC: Stem Cell; STAT: Signal Transducer and Activator of Transcription; SOCS: Suppressor of Cytokine Signaling; TEPs: Thiolester Proteins; Upd: Unpaired.



Yet another role for JNK signaling in *Drosophila* is to protect from oxidative stress by activating the expression of several autophagy-related (ATG) genes [36]. The ATG genes are required for the oxidative stress-protection function of the JNK pathway in the *Drosophila* intestinal epithelium [36].

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However, only stress-induced autophagy is dependent on JNK signaling [36]. Thus, JNK-mediated expression of ATG genes, can increase the resistance to oxidative stress, but it remains unclear if the same genes also have a role in longevity [36]. The transcription factor(s) downstream of the JNK pathway that mediate the activation of ATG genes are unknown, although FOXO may be one of them [36].

Drosophila eiger, the sole homolog of the tumor necrosis factor (TNF) and signaling through its receptor *wengen* (TNF receptor homolog), is suggested to play opposing roles in the fly's response to infection [37]. This is because there are two TNF receptor-associated factors in *Drosophila*, TRAF1 and 2. The association of each one of them with the receptor *wengen* leads to the activation of different signaling cascades [38]. When TRAF1 associates with *wengen*, apoptosis ensues [38]. On the other hand, the association of TRAF2 with the receptor *wengen* leads to Imd signaling activation and AMP expression [38] (Figure 1).

Strikingly, induction of innate immune response and the Ras oncogene expression in the *Drosophila* hindgut result in the invasion and dissemination of oncogenic hindgut epithelial cells [39]. In the hindgut, bacterial infection induces the Imd pathway, which synergizes with the Ras oncogene to induce the JNK signaling and MMP1 expression. MMP1in turn degrades the extracellular matrix, leading to cell invasion and dissemination [39]. Noticeably, the Imd-JNK-MMP1 pathway in Ras-activated hindgut cells is also activated in immune challenged *Drosophila* hemocyte-like cells [40], suggesting a direct link between epithelially emanating inflammatory signals and cancer cell migration.

3.2. Mammalian Epithelial Immune Responses and Parallels with Drosophila

In mammals, TNF receptor (TNFR), Toll-like receptor (TLR), as well as phagocytic activities, activate Nox (NADPH oxidases) enzymes leading to ROS production [35,41] (Figure 1). In the colonic and other human epithelia, the Nox family oxidases, Nox1 and Duox2, are expressed, playing a critical role in chronic inflammation [42–45]. The human Duox2 is an ortholog of the *Drosophila* Duox [42]. Although the mechanism by which TLRs mediate Nox/Duox-dependent ROS production is not clear [35], TLR4-mediated ROS production is required for the activation of the TRAF6-ASK1-p38 pathway to alert cells of infection [46]. Likewise, in tumor necrosis factor TNFα signaling, ROS generation is needed for the activation of ASK1 by TRAF2 and sustained JNK/p38 activation for the induction of apoptosis [46]. Similarly to *Drosophila*, mammalian G-protein-coupled receptors (GPCR) can induce NADPH oxidases (e.g., Nox) leading to ROS production [47] (Figure 1). Moreover, the mammalian Prx family enzymes are necessary for eliminating ROS in order to protect cells from oxidative cytotoxicity. This process takes place during the activation and the secretory activity of macrophages [48,49].

Moreover, Crohn's disease, which is a type of inflammatory bowel disease (IBD), is associated with autophagy [50,51]. In patients with Crohn's disease, single-nucleotide polymorphisms (SNPs) were found in autophagy-related genes [50]. Specifically, SNPs have been identified in the autophagy gene ATG16L1, in autophagy-stimulatory GTPase IRGM and in NOD2, which is an intracellular bacterial sensor [50,52]. Normally, NOD2 recognizes the bacterial PGN-derived muramyl-dipeptide (MDP) and recruits ATG16L1, thus inducing autophagy [53]. Importantly, a mutation in the Crohn's disease susceptibility gene Atg16L1 promotes several hallmarks of the Crohn's disease upon viral infection [51]. Furthermore, Crohn's disease is associated with microRNAs that negatively regulate IRGM and autophagy [52,54]. Thus, similar to the induction of autophagy-related (ATG) genes in *Drosophila*

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upon oxidative stress, the properly set expression of ATG genes in mammals is crucial for epithelial homeostasis.

4. Epithelial ISC Responses: Regenerative Inflammation

Cytokines are low-molecular weight polypeptide proteins that are mainly known for their role in immune response and inflammation [55,56]. They are secreted molecules usually acting in the producing (autocrine mode) or adjacent cells (paracrine mode) [57]. Interleukins (IL), interferons (IFN) and tumor necrosis factor (TNF) are the main cytokines [58]. An induced cytokine is able to stimulate the production of many other specific cytokines, in order to create a network of cooperating molecules [55]. However, abnormal levels of cytokines or their receptors results in serious pathologies, such as chronic inflammatory diseases and cancers [56,59]. For example, TNF as well IL-1 and IL-6 are inducible by hypoxia, a hallmark of tumor cells [3]. These cytokines act to suppress cell death, activate stem cells, and promote epithelial proliferation at the site of the injury [60]. Some cytokines may also act as autocrine growth factors to promote the survival of malignant cells [3]. Indeed, IL-6 acts as growth factor for hematological malignancies and IL-1 for gastric carcinoma [3].

Growth factors are also polypeptides that usually induce cell growth or proliferation and may have overlapping functions with cytokines in both *Drosophila* and mammals (Table 1). In the following sections, we discuss the growth factor- and cytokine-induced signaling pathways that control the intestinal stem cells maintenance in both mammals and *Drosophila*, thereby emphasizing the overlap in function between growth factors and cytokines during intestinal "regenerative inflammation" and cancer.

4.1. Growth Factors and Cytokines in Intestinal Stem Cell Maintenance

Adult intestinal stem cells (ISC) are present in the *Drosophila* and the mammalian intestine and are responsible for the maintenance of intestinal homeostasis by continuously replacing the intestinal cells, a process mediated by conserved signaling pathways [5,61,62]. The *Drosophila* midgut ISCs are located basally within the intestinal epithelium and give rise to nutrient-absorbing enterocytes and enteroendocrine cells, two differentiated cell types also found in mammals [5]. Before differentiation, ISC are asymmetrically divided into an enteroblast cell and a self-renewing stem cell. The enteroblast differentiates into an enterocyte or enteroendocrine cell depending on the levels of Delta protein, which is the ligand of the Notch signaling pathway [63,64]. In the mammalian intestine, Notch signaling is also required for ISC self-renewal and fate decisions [65]. Notch signaling that promotes ISC proliferation in the mouse intestine, requires cooperation with Wnt, a growth factor also necessary for *Drosophila* ISC maintenance and proliferation [66,67].

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Table 1. Mammalian and *Drosophila* homologous cytokines and growth factors in ISC proliferation and differentiation and EC apoptosis and immune response during homeostasis or infection/stress of the intestine. The first column shows biological processes conserved between *Drosophila* and mammals, while the other two columns represent the corresponding cytokines and growth factors for each species. The homologous components are placed on the same line. The parentheses show critical components of the pertinent signaling pathways in which each cytokine and growth factor are major players. ISC: Intestinal Stem Cells; EC: Enterocytes; PAMPS: Pathogen-Associated Molecular Patterns (e.g., peptidoglycan).

	Mammals	***Drosophila***
ISC Proliferation/ Maintenance	Wnt IL-6 (Stat3) EGF (EGFR-Ras)	Wingless Upds (JAK/STAT) Spitz,Keren,Vein (EGFR-Ras1)
ISC Differentiation	Ihh BMP Wnt IL-6 (Stat3) EGF (EGFR-Ras)	Hh Dpp? Wingless Upds (JAK/STAT) Spitz,Keren,Vein (EGFR-Ras1)
EC Apoptosis	TNF (PAMPs/NF-κB/JNK)	Eiger (PAMPs/NF-κB/JNK)
EC Immune Response	TNF (PAMPs/NF-κB/JNK) IL-6 (Stat3)	Eiger (PAMPs/NF-κB/JNK) Upds (JAK/STAT)

Intestinal stem cells also exist in the posterior intestine of *Drosophila*, the hindgut, but they are confined in the *hindgut proliferation zone* (HPZ). Within this zone, ISC self-renewal, proliferation and differentiation, are controlled by Wingless and Hedgehog (Hh) signaling [68]. Specifically, the Hh signaling pathway is required for the transition of ISC from the proliferative to the differentiation state [68]. Similarly, in the crypt epithelium of the mammalian intestine, where ISCs reside, the Wnt and Hh pathway ligands are expressed [68]. Indian Hedgehog (Ihh) induces the formation and proliferation of the mesenchymal cells, which in turn regulate the proliferation and differentiation of nearby ISCs [69]. Wnt signaling is critical for the maintenance of murine intestinal stem cells and progenitors, as it has been shown in mice lacking β-catenin, a positive effector of the Wnt pathway [70]. The blocking of Wnt/β-catenin signaling results in rapid loss of crypts and cell proliferation and terminal differentiation of intestinal stem cells [70]. The opposite phenotypes (increased proliferation, crypt expansion and decreased differentiation) were observed, in experiments with overexpressed β-catenin [70]. Additionally, several signals including the Bone Morphogenetic Protein (BMP) antagonize Wnt signaling to the crypts [61]. Bone Morphogenetic Proteins (BMP) belong to the transforming growth factor beta (TGF-b) superfamily and negatively regulate the ISC proliferation [71]. BMP signaling is maintained by Ihh signals, in order to promote the differentiation of epithelial and mesenchymal cells in the villus [61]. However, in the crypt, there is a production of BMP antagonists by the myofibroblasts, in order to inhibit the BMP signaling, thus maintaining the Wnt activities [61].

Loss of function of the Wg pathway in the *Drosophila* midgut does not lead to rapid ISC loss to support the idea that Wg signaling is the main regulator of ISC self-renewal and differentiation, as in the mammalian model [72]. Instead, the Wg, JAK/STAT and EGFR/Ras/Erk signaling pathways

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cooperate to maintain ISCs [73]. Thus, the simultaneous disruption of all three of them results in complete elimination of ISC in a short time, while disruption of a single one can be replaced by over-activation of one of the other two [73].

Wg and the EGFR signaling pathway ligand Vein are expressed in the *Drosophila* visceral muscle, which acts as a stem cell niche [72–74]. JAK/STAT pathway is induced by ligands emanating from the visceral muscle [73] or the intestinal epithelium cells [75]. On the other hand, Notch signaling represses transcriptionally JAK/STAT signaling ligand unpaired (upd) [76]. Conversely, JAK/STAT antagonize Notch signaling during enteroblast fate decision [77]. High levels of JAK/STAT signaling leads to differentiation into enteroendocrine cells, while low levels of its activation, preferentially lead to differentiation into enterocytes [77].

Three of the pathways that regulate ISC maintenance in *Drosophila*, are also induced by niche signals that are provided by the mammalian Paneth cells, including EGF (EGFR ligand), Wnt3 (Wnt ligand) and Dll4 (Notch ligand) [78]. Thus, Paneth cells have been characterized as "multifunctional guardians" of the mammalian intestinal stem cells [78]. Nevertheless, sub-epithelial myofibroblasts are also proposed to maintain mouse ISCs [79]. The overall process is strikingly similar to the *Drosophila* cytokines and growth factors emanating from both the epithelium [75] and the visceral muscle [73].

Interestingly, the *Drosophila* midgut and the mammalian intestine share similarities in the symmetry of ISC divisions. Recent studies, suggest that 2 out of 10 ISC divisions in the *Drosophila* midgut, are symmetric to balance for the occasional loss of ISCs [80,81]. This is similar to the Lgr5+ mammalian ISCs, which compensate for stem cell loss by symmetric division [82].

4.2. Intestinal Epithelium Regeneration and Cancer-Promoting Inflammation

The *Drosophila* midgut epithelium is a dynamic tissue, capable of regenerating the whole intestinal epithelium when damaged or infected by expressing growth factors and signals that promote ISC proliferation and differentiation. Over the years, many studies indicate inflammation and immune signals as enabling characteristics of cancer, although the connection between inflammation and cancer is not fully elucidated [2]. In mammals, stressed or dying cells due to infection promote inflammation by the activation of different types of immune cells e.g., macrophages, neutrophils, T-cells and B-cells, which in turn activate a variety of tumor-promoting inflammatory cytokines (Figure 3) [60]. Extensive chronic tissue damage and cell death perpetuates inflammation and regeneration by an increase in stem cell proliferation, in addition to a higher probability in harboring oncogenic mutations [60]. Inflammatory cytokines are also expressed by cancer cells, which in turn recruit immune cells leading to tumor-associated inflammation [60]. Most importantly, infiltrating blood cells in the tumor microenvironment are traditionally believed to be the instigators of tumor-promoting inflammation [2,3].

In contrast to mammals, the *Drosophila* intestine does not appear to be infiltrated by hemocytes (*Drosophila* phagocytes [83]) in response to infection. Although phagocytosis by hemocytes is crucial in fighting intestinal infections when bacteria escape from the intestine into the hemolymph e.g., upon infection with the entomopathogenic bacterium *Serratia marcescens* [84], this process does not lead to the infiltration of hemocytes into the epithelium [83,84]. On the contrary, *Drosophila* intestinal epithelium-emanating pro-inflammatory signals (cytokines and growth factors) can directly promote ISC proliferation and differentiation and regenerate the damaged epithelium. This "regenerative inflammation"

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is a dynamic process mainly controlled by at least four evolutionary conserved signaling pathways (Figure 3). Two of them are the JNK and the Hippo signaling pathways, which are activated as a consequence of intestinal epithelium infection in order to firstly induce the production of cytoprotective genes (JNK) or in damaged or stressed cells to induce ISC proliferation and regeneration (JNK and Hippo) [85]. JNK and Hippo signaling promotes the expression of IL-6-like pro-inflammatory cytokines *unpaired (upd)*, *unpaired 2 (upd2)* and *unpaired 3(upd3*) by the damaged midgut epithelium cells, as well the secretion of EGFR signaling pathway-ligands promoting over-proliferation of ISC and intestinal hyperplasia [5,85,87]. Moreover, upon bacterial infection Upd3, which is released by the enterocytes, seems to have an additive effect with Upd2 in the induction of epithelial regeneration [75].

The other key player in *Drosophila* midgut epithelium regeneration is the EGFR/Ras/MAPK pathway. The epidermal growth factor receptor (EGFR) is activated by three EGF ligands: Spitz and Keren (produced within the intestinal epithelium) and Vein, which is produced by the visceral muscles [86,88]. Induction of Vein in the visceral muscles requires the activation of the JAK/STAT pathway by the Upd3 cytokine, which is expressed by damaged enterocytes [86]. Upd3 can also induce Spitz in enteroblasts [88]. Independently of their source, these EGFR pathway ligands induce ISC proliferation and midgut hyperplasia [85].

More recent studies show that Wg is another damage-inducible pathway, which is required for ISC proliferation during *Drosophila* midgut regeneration [89]. Wg is also an important target of the JNK signaling. The activation of JNK in ECs upon intestinal damage or stress, results in the secretion of Wg by the EBs and the activation of Wg signaling, which in turn upregulates Myc in the ISCs and leads to their proliferation [89]. Importantly, regeneration, but not ISC self-renewal, requires Wg expression in the midgut enterocytes [89].

Various bacterial pathogens can activate the aforementioned conserved signaling pathways to induce regeneration of the damaged or stressed *Drosophila* intestine. For example, *Drosophila* infection with high doses of *Pseudomonas entomophila* induces epithelium renewal while even higher concentration of the same bacterium leads to ISC loss [90]. The JNK and JAK-STAT pathways are induced in the gut cells upon infection with *P. entomophila*, *Erwinia carotovora carotovora 15* (Ecc15), *Serratia mascescens* and *Pseudomonas aeruginosa* [90]. Interestingly, when bacterial infection is combined with low cytologically innocuous expression levels of an oncogenic form of Ras1 gene (ortholog of the mammalian K-Ras) in the midgut ISCs and progenitors, intestinal dysplasia ensues. This is due to a synergism between the bacterial infection-induced JNK and the Ras oncogene [40,91]. In this model, the virulent strain (PA14) of the human opportunistic pathogen *P. aeruginosa* damages and induces regeneration of the epithelium *via* JNK signaling [91]. In contrast, the avirulent (CF5) *P. aeruginosa* strain causes no damage in the midgut epithelial cells [91]. The virulence factor *pyocyanin* secreted by the virulent but not by the avirulent *P. aeruginosa* contributes to the ISC over-proliferation during infection [91].

The capability of pathogenic bacteria to induce cancer initiation and progression was also examined in mammalian models. Recently, *Escherichia coli* was shown to induce intestinal tumorigenesis and inflammation in mice. *E. coli* strain NC101 harboring a polyketide synthase (pks), which is a DNA-damaging toxin, known as colibactin, is required for the progression of colorectal cancer (CRC) in carcinogen-treated interleukin-10 deficient mice [92]. Importantly, NC101 was detected in 40% of inflammatory bowel disease (IBD) patients and in almost 70% of CRC patients, indicating pks as a

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potential tumor-promoting factor [92]. This and other studies in mammals show that apart from intestinal damage and inflammation, the genotoxicity are properties of some bacteria that may promote human CRC. Consistently, a hypothetical model proposed by Ben-Neriah and Karin links ROS and nitric oxide (NO) production with mutagenesis of critical genes in the intestinal stem cells, such as the gene encoding adenomatosis polyposis coli (APC), resulting in an adenoma growth and colorectal tumor generation [93].

Noticeably, the majority of the signaling pathways that contribute to the *Drosophila* regenerative inflammation may also contribute to tumor initiation and progression in mammals. The mammalian JAK/STAT pathway signaling requires NF-κB activation for the production of pro-inflammatory cytokines and growth factors during colitis-associated cancer (CAC) [94]. Interleukin-6 (IL-6) is one NF-kB-dependent cytokine, which induces the oncogenic transcription factor STAT3 in order to promote proliferation and survival of tumor-initiating intestinal epithelial cells, thus contributing to CAC tumorigenesis in mice [94]. Importantly, the cytokine IL-6 acts not only in epithelial but also in immune cells and is produced by the lamina propria, a layer of connective tissue, which does not exist in the *Drosophila* intestine [5,94]. Lamina propria is located under the intestinal epithelium and, together with the epithelium, houses many immune cells e.g., macrophages, dendritic cells and B-cells [5]. The absence of this layer in the *Drosophila* intestine correlates with the absence of immune cells in the *Drosophila* intestine, but further studies are required to clarify if *Drosophila* hemocytes play any role in intestinal inflammation.

Regardless, two ligands of the mammalian EGFR pathway, amphiregulin (AREG) and epiregulin (EREG), are induced by the pro-inflammatory cytokines IL-1β and TNF-a in inflamed colonic mucosa and in adenomas and carcinomas of human colon, but not in normal colonic mucosa [22]. Importantly, in patients with ulcerative colitis and Crohn's disease, the epithelial cells rather than the mesenchymal cells, exhibit high expression of amphiregulin and epiregulin [22]. Thus an auxiliary mechanism of inflammation, similar to that observed in the *Drosophila* intestine may exist in the human intestine, where pro-inflammatory signals and growth factors emerging from the inflamed colonic epithelial cells and the tumors may act directly in a paracrine manner to facilitate intestinal stem cell proliferation and tumor progression. Furthermore, epithelial cells surrounding colon cancer stem cells secrete a hepatocyte growth factor (HGF) and maintain high Wnt activity in colon cancer stem cells, but also induce the activation of Wnt in differentiated cancer cells [95]. Pattern recognition receptors e.g., Toll-like receptors (TLR) are also activated in epithelial cells during tumorigenesis by oxidative stress, bacterial products and tissue damage [96]. These findings suggest that the epithelial microenvironment may contribute significantly to the propagation of the colon cancer cells [95].

In infected *Drosophila*, the Toll pathway activity appears to limited to the systemic immune response *i.e.*, in the fat body and hemocytes, where Toll acts as an immune sensor [97]. In contrast, the Imd pathway is activated both systemically and in the midgut and hindgut epithelium [97]. In the infected midgut epithelium, Imd acts in a p38-dependent manner to regulate ROS production and in a Relish-dependent manner for the local expression of AMPs [29,97,98]. Whether NF-κB-mediated immune response in flies is linked to the regeneration process is still an open question.

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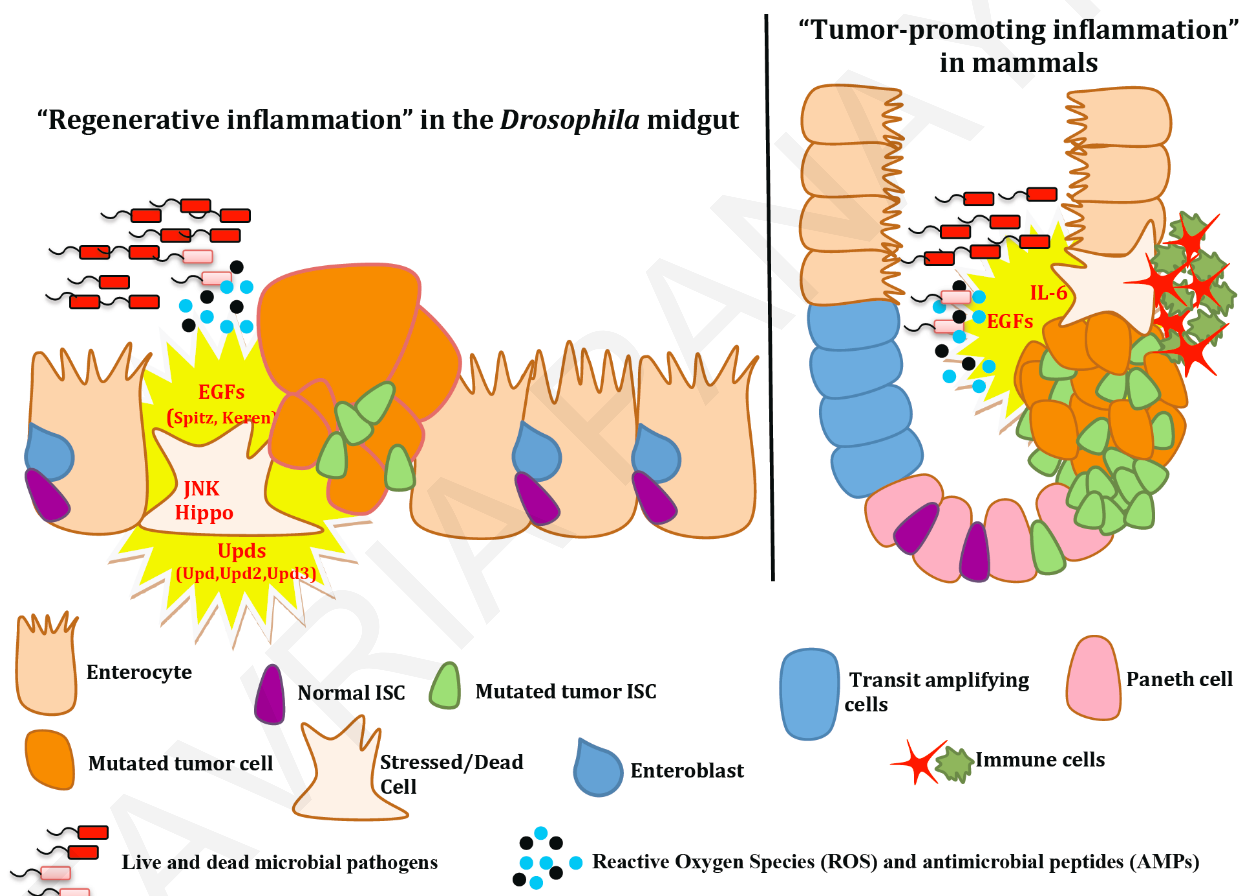
Figure 3. "Regenerative inflammation" in the *Drosophila* midgut resembles but also differs from "tumor-promoting inflammation" in mammals. Upon intestinal bacterial infection in *Drosophila*, growth factors (EGFs) and cytokines (Upds: IL-6-like cytokines) are secreted by the damaged epithelial cells and activate ISC proliferation and differentiation in order to regenerate the damaged midgut epithelium. Homologs of these growth factors and cytokines have also been observed in mammalian tumor-initiating epithelial cells. In contrast to the *Drosophila*, "regenerative inflammation", which is directly induced by epithelially emanating signals, the mammalian "tumor-promoting inflammation" includes the infiltration of blood cells at the place of the damage and ISC proliferation. Reactive oxygen species (ROS) are also activated in both species and have opposing roles upon damage: they help the host to fight the infection, but they may also contribute to mutagenesis and tumor formation.



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5. Other Frontiers in *Drosophila* Inflammation

5.1. Organ Communication: Inflammatory Signal Cross-Talk between Different Organs

Drosophila larvae hemocytes communicate with the fat body upon septic injury and oral infection [99]. In addition, cytokines control AMP expression in the larval fat body in a process that strongly resembles the mammalian response to bacterial infection [99]. This is mediated by the Toll ligand Spätzle, a cytokine secreted by the hemocytes [96]. Thus, Spätzle's knockdown in the larvae hemocytes blockes the expression of the AMP gene *drosomycin*, in the fat body [96]. Furthermore, a

lysosomal protein Psidin has a dual role in the immune response upon infection in the detection of the bacteria by the hemocytes, and in the activation of the AMP gene *defencin* in the fat body [100]. Moreover, *Drosophila* Upd3 expression in hemocytes induces JAK/STAT signaling in the fat body, in order to activate the expression of *totA* peptide, which is suggested to be a general stress-response factor [10,101]. Interestingly, *totA* also requires the Imd/Relish signaling, which is activated in response to many Gram-negative bacteria, in the fat body cells [10]. These findings suggest that, apart from their phagocytic activities, the hemocytes of the *Drosophila* larvae also act through a cytokine-based regulatory signal, similar to mammalian innate immune response, which comprises the release of cytokines and chemokines by activated immune cells (e.g., macrophages) upon bacterial infection [99].

In a tumor model (Ras^{V12}/$scrib^{-/-}$) of eye–antennal imaginal discs of *Drosophila*, larval hemocyte number increases through the activation of the JAK/STAT pathway [102]. The STAT transcription factor is highly induced in these tumors, and in the circulating hemocytes, but not in the wild-type larvae [102]. JAK/STAT is also activated in mechanically wounded larvae discs. Furthermore, the local activation of the JNK pathway induces the expression of JAK/STAT ligands (unpaired cytokines) in both tumors and wounds [102]. This model suggests that JNK signaling in the damaged tissue induces the expression of Upd ligands and the subsequent activation of the JAK/STAT pathway in the hemocytes and the fat body, resulting in additional cytokine expression, and as a consequence an increase in the hemocytes number [102].

Organ-to-organ communication also occurs between *Drosophila* gut, hemocytes and the fat body, during the larval innate immune response [103,104]. Upon bacterial infection nitric oxide (NO) is produced in the gut, while the hemocytes, which are activated *via* the NO-depended signal, function as an intermediary in order to pass the signal to the fat body, resulting in the production of the AMP *Diptericin* [103,104]. Activation of the Rel/NF-κB pathway in the fat body cells is required for AMP production [103,104].

5.2. Intestinal Microbiota and Inflammation

The characterization of *Drosophila* gut microbiota and the capability of some bacteria to produce cancer-related phenotypes in synergy with the genetic predisposition in *Drosophila* intestine, as well the high conservation of the mechanisms and the signaling pathways that regulate ISC maintenance and innate immunity between *Drosophila* and mammals, have made *Drosophila* an attractive model for understanding the interactions occurring between the microbiota and the human gut, as well their potential role in gut pathogenesis, inflammation and cancer.

There are usually about 1 to 20 different species of bacteria in the *Drosophila* gut, while in the mammalian intestine, there are at least hundreds of different species. *Drosophila* microbiota is therefore much simpler [105,106]. In *Drosophila*, only a few aerotolerant bacteria species are found e.g., *Lactobacillus* species, while the strictly anaerobic species such as *Bacteroidetes* that are abundant in the human flora, are absent the *Drosophila* intestine [105]. *Lactobacillus plantarum* and *Enterococcus faecalis* were recently tested for their ability to colonize germ-free *Drosophila*. Although both of them can colonize young larvae, only *L. plantarum* is considered innocuous or beneficial and remains associated with *Drosophila* long after initial colonization [107]. Indeed, several strains of

L. plantarum stimulate larval development upon nutrient scarcity and adults emerge faster than in the germ-free animals [107]. *E. faecalis* is another common colonizer of the human bowel [108]. Nevertheless, virulent *E. faecalis* strains produce cytolysin, which has a dual role both as a toxin and a bacteriocin [108]. Flies feeding on a virulent, cytolysin toxin-expressing *E. faecalis* strain exhibit significantly increased lethality [108]. Additionally, a virulence determinant possessing homology to many human pathogenic bacteria, termed KerV, is crucial for the pathogenesity of several bacterial species [109]. On of them is *P. aeruginosa*, a principal agent of lethal infections in cystic fibrosis, severely wounded and cancer patients [110]. Two more are *Vibrio cholera*, the etiological agent of cholera and *Yersinia pseudotuberculosis*, a gastrointestinal pathogen, both of which require the kerV gene to exert full virulence upon introduction in the adult *Drosophila* intestine [109]. In addition, host metabolism can be modified by commensal bacteria, such as *Acetobacter pomorum* in *Drosophila*. *A. pomorum* modulates insulin/insulin-like growth factor signaling, which in turn affects the developmental rate, the metabolism and the intestinal stem cell activity [111].

Host genes also shape intestinal microbiota. In *Drosophila* gut epithelia, the caudal protein is required in order to repress NF-κB-dependent AMP expression and, in turn, maintain a balanced flora community [98]. In caudal knockdown flies, there is an overexpression of AMPs, which results in an unbalanced community structure (dysbiosis) and a subsequent elevated gut apoptosis and host death [98]. Inflammatory bowel diseases also involve apoptosis of human intestinal cells. Thus perturbations in the intestinal NF-κB pathway may be relevant to the etiology of intestinal pathogenesis in both mammals and flies [112]. Furthermore, it was demonstrated that dual oxidase (Duox) activity in the fly gut is controlled by multiple Duox-regulatory signaling pathways, which "fine-tuned" ROS production depending on the type of gut–microbe interactions [113]. That is, negative regulation of Duox occurs in response to colonization with commensal microbes while, if infectious microbes colonize the gut, there is a positive regulation of Duox [113].

In conclusion, gut microbiota provide protective, metabolic and nutritional signals and help the host to ward off harmful microbes that elicit intestinal damage and concomitant inflammation [114]. Therefore, in order to maintain a healthy intestinal epithelium, a harmonious coordination of the gut microbiota, immune and stem cell responses, and environmental factors such as diet, is required [90]. If the delicate balance between these factors breaks, inflammatory diseases may develop.

6. Conclusions

During the last 20 years, *Drosophila* has provided invaluable insights in the field of innate immunity. It helped tremendously to decipher the mammalian innate immune responses and for that, a *Drosophila* scientist, Jules Hoffman, was co-awarded the Nobel Prize in Physiology or Medicine in 2011. While the conservation of innate immune responses between insects and mammals is now literally textbook knowledge, cytokine and growth factor signaling pathways have been recently shown to induce epithelial immunity and regeneration that facilitates cancer-related phenotypes. Contrary to mammals, this "regenerative inflammation" does not require *Drosophila* hemocytes in order to induce ISCs and predispose for tumor formation. This apparent discrepancy might be due to physiologic differences between flies and mammals e.g., the lack of lamina propria in flies where mammalian blood cells accumulate upon inflammation. Nevertheless, studies on *Drosophila* "regenerative inflammation"

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might help to decipher the role of epithelially emanating cytokines and growth factors in ISC induction in the absence of blood cell infiltration. Because chronic inflammation, while irrefutably a major driver of carcinogenesis, manifests itself in only a subset of cancers [3], we believe that even in the absence of blood cell infiltration, increased intestinal regeneration propelled directly from epithelial cytokines and growth factors might be the instigator of a more broadly defined inflammation-driven carcinogenesis.

Conflict of Interest

The authors declare no conflict of interest.

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