**Importance of flagella in acute and chronic
Pseudomonas aeruginosa infections.**

Item Type	Article
Authors	Lorenz, Anne; Preuße, Matthias; Bruchmann, Sebastian; Pawar, Vinay; Grahl, Nora; Pils, Marina C; Nolan, Laura M; Filloux, Alain; Weiss, Siegfried; Häussler, Susanne
DOI	10.1111/1462-2920.14468
Publisher	Wiley-Blackwell
Rights	embargoedAccess; Attribution-NonCommercial-ShareAlike 4.0 International
Download date	09/03/2020 13:15:18
Item License	http://creativecommons.org/licenses/by-nc-sa/4.0/
Link to Item	http://hdl.handle.net/10033/621615

1Importance of flagella in acute and chronic *Pseudomonas aeruginosa* infections

2

3Anne Lorenz^{1,4¶}, Matthias Preuße^{1,4¶}, Sebastian Bruchmann^{1,2,4}, Vinay Pawar^{1,3}, Nora Grahl^{1,4},

4Marina C. Pils⁵, Laura M. Nolan⁶, Alain Filloux⁶, Siegfried Weiss^{3&}, Susanne Häussler^{1,4&*}

5¹Institute of Molecular Bacteriology, Helmholtz Centre for Infection Research, Braunschweig,

6Germany

7²Wellcome Sanger Institute, Cambridge, United Kingdom

8³Institute of Immunology, Medical School Hannover, Hannover, Germany

9⁴Institute of Molecular Bacteriology, TWINCORE GmbH, Center for Clinical and

10Experimental Infection Research, Hannover, Germany

11⁵Mouse Pathology, Animal Experimental Unit, Helmholtz Centre for Infection Research,

12Braunschweig, Germany

13⁶MRC Centre for Molecular Bacteriology and Infection (CMBI), Department of Life

14Sciences, Imperial College London, London, United Kingdom

15* corresponding author

16E-mail: Susanne.Haeussler@helmholtz-hzi.de

17[¶]These authors contributed equally to this work

18[&]These authors contributed equally to this work

19Running title: Flagella expression in acute and chronic infections

20Originality-significance Statement

21*Pseudomonas aeruginosa* is an opportunistic pathogen, a causative agent of a wide range of
22devastating acute, but also chronic persistent infections. Given this remarkable versatility, an
23important question is whether the environmental cues drive the initiation of an acute virulent
24versus a chronic biofilm formation program by *P. aeruginosa*. This ultimately determines
25very diverse disease outcomes. Here, we have used *in vivo* selection of transposon mutant
26libraries in mouse models of acute or biofilm-associated infections. We demonstrate that
27expression of flagella was negatively selected during acute *P. aeruginosa* lung infections. In
28contrast, flagella play a key role in the establishment of biofilm-associated infections.
29Apparently, a complex interplay between adaptive bacterial behavior and the host defense
30exists. It seems that the host response to the infectious agent rather than the activation of
31distinct pathogenic traits of the bacteria is a major driver for acuteness or duration of the
32infection. This work should be of high interest to the readers of Environmental Microbiology.
33It contributes to our understanding of the establishment of biofilm-associated infectious
34disease. The finding that the bacteria apparently form biofilms as an adaptive response to the
35immune reactions of the host has serious implications for the prevention and treatment of
36chronic persistent infections.

37

38Summary

39*Pseudomonas aeruginosa* is an environmental microorganism and a causative agent of diverse
40acute and chronic, biofilm-associated infections. Advancing research-based knowledge on its
41adaptation to conditions within the human host is bound to reveal novel strategies and targets
42for therapeutic intervention. Here, we investigated the traits that *P. aeruginosa* PA14 as well
43as a virulence attenuated $\Delta lasR$ mutant need to survive in selected murine infection models.
44Experimentally, the genetic programs that the bacteria use to adapt to biofilm-associated
45versus acute infections were dissected by passaging transposon mutant libraries through
46mouse lungs (acute) or mouse tumors (biofilm-infection). Adaptive metabolic changes of
47*P. aeruginosa* were generally required during both infection processes. Counter-selection
48against flagella expression was observed during acute lung infections. Obviously, avoidance
49of flagella-mediated activation of host immunity is advantageous for the wildtype bacteria.
50For the $\Delta lasR$ mutant, loss of flagella did not confer a selective advantage. Apparently, other
51pathogenesis mechanisms are active in this virulence attenuated strain. In contrast, the
52infective process of *P. aeruginosa* in the chronic biofilm model apparently required
53expression of flagellin. Together, our findings imply that the host immune reactions against
54the infectious agent are very decisive for acuteness and duration of the infectious disease.
55They direct disease outcome.

56Introduction

57The ability to thrive in diverse aquatic and terrestrial habitats is a hallmark of the
58metabolically versatile environmental bacterium *Pseudomonas aeruginosa* (Bjarnsholt *et al.*,
592013; Folkesson *et al.*, 2012; Klockgether & Tümmler, 2017). At the same time,
60*P. aeruginosa* is a successful pathogen that causes a wide spectrum of opportunistic
61infections in a broad range of different hosts (Dubern *et al.*, 2015; Rahme *et al.*, 1995, 2000).
62In general, two types of clinical manifestations can be distinguished: an acute, often life-
63threatening course of infections (Chatzinikolaou *et al.*, 2000; Crouch Brewer, Wunderink,
64Jones, & Leeper, 1996; Nathwani, Raman, Sulham, Gavaghan, & Menon, 2014; Osmon,
65Ward, Fraser, & Kollef, 2004) and a chronically persistent, progressive course, involving
66biofilm-formation (Bjarnsholt, 2013; McManus, Mason, McManus, & Pruitt, 1985; Wagner
67& Iglewski, 2008). *P. aeruginosa* rapidly and efficiently adapts to challenging habitats, e.g.
68the respiratory tract of cystic fibrosis patients (Breidenstein, de la Fuente-Núñez, & Hancock,
692011; D'Argenio *et al.*, 2007; Hancock *et al.*, 1983; Häussler, Tümmler, Weissbrodt, Rohde,
70& Steinmetz, 1999; Luzar & Montie, 1985; Mahenthiralingam, Campbell, & Speert, 1994;
71Oliver, Cantón, Campo, Baquero, & Blázquez, 2000; E. E. Smith *et al.*, 2006). More effective
72intervention strategies to combat these devastating acute as well as therapy-refractory chronic
73infections are an important goal of biomedical research. Steps towards this goal require
74knowledge on mechanisms driving the different clinical manifestations of *P. aeruginosa*
75infections.

76A selection of individual genes of *P. aeruginosa* is expected to be required for survival in
77particular habitats. These can be efficiently assessed, by *in vivo* selection of transposon
78mutant libraries in combination with insertion tracking by deep sequencing (Kwon, Ricke, &
79Mandal, 2016). Relative frequencies of transposon (Tn) insertions in input pools of mutant

80libraries compared to output pools, that have been passaged *in vivo*, can be determined in a
81high throughput manner. By using murine models, such Tn sequencing studies have
82uncovered the role of gene products of *P. aeruginosa* that are involved in the establishment of
83infections in the agar bead lung infection model ((Bianconi *et al.*, 2011; Potvin *et al.*, 2003),
84bacterial colonization of the mouse intestine (Goodman *et al.*, 2004; Roux *et al.*, 2015) and
85its subsequent systemic dissemination (Skurnik, Roux, Aschard, *et al.*, 2013; Skurnik, Roux,
86Cattoir, *et al.*, 2013). Furthermore, mouse wound infections have been shown to be suitable
87systems to study the contribution of individual *P. aeruginosa* genes to the establishment of
88chronic versus acute soft tissue infections (Turner, Everett, Trivedi, Rumbaugh, & Whiteley,
892014).

90In the present study we passaged Tn mutant libraries of *P. aeruginosa* PA14 and the virulence
91attenuated $\Delta lasR$ deletion mutant in two established *in vivo* systems. First, we used a mouse
92model of acute pneumonia, where we instilled bacterial cells intra-tracheally. Alternatively,
93we infected solid tumors of mice with the Tn libraries. We had shown before that bacteria
94colonize the tumor tissue upon intravenous infection and rapidly establish biofilm structures.
95Thus this tumor model is perfectly suited to study requirements for the initiation of biofilm
96formation in an infected host. According to the gene expression profile of *P. aeruginosa*, the
97bacteria recognize this niche as a habitat similar to cystic fibrosis lungs (Bielecki *et al.*,
982011). Consequently, residence of the bacteria in this neoplastic tissues leads to development
99of therapy-refractory biofilm infections (Crull *et al.*, 2011; Komor *et al.*, 2012; Pawar *et al.*,
1002015b). Comparison of the *in vivo* selected mutant pools from both systems implies that the
101immune response of the host against the infectious agent rather than the expression of
102bacterial virulence traits modulates disease outcome and drives acuteness and duration. Our
103results highlight the value of functional genomics approaches to study bacterial strategies
104responsible for acute versus biofilm-associated infections.

105Results

106*P. aeruginosa* PA14 wildtype and the virulence attenuated *lasR* mutant in mouse models 107of acute and biofilm-associated infections

108We wanted to determine the contribution of non-essential genes of *P. aeruginosa* to overall
109fitness in acute versus biofilm-associated infections. Therefore, we tested a previously
110generated PA14 wildtype (WT) Tn mutant library (Skurnik, Roux, Aschard, *et al.*, 2013) in
111two *in vivo* systems. Similarly, a *lasR* Tn mutant library, which has been generated in this
112study, was employed. LasR is a global quorum sensing regulator, which governs the
113production of a large array of virulence factors (Feltner *et al.*, 2016; J. Lee & Zhang, 2015;
114Lima *et al.* 2018; Yan *et al.* 2018). Mutants of *lasR* are attenuated in several models of acute
115infections (Lesprit *et al.*, 2003; Lorè *et al.*, 2012; Rumbaugh, Griswold, Iglewski, & Hamood,
1161999; Tan, Rahme, Sternberg, Tompkins, & Ausubel, 1999; Tang *et al.*, 1996). Interestingly,
117despite the important role of LasR in quorum sensing and its regulation of virulence factors,
118isolates of *P. aeruginosa* that bear mutations in the *lasR* gene are frequently recovered from
119the chronically infected respiratory tract of cystic fibrosis patients (Cabrol *et al.*, 2003;
120Feltner *et al.*, 2016; Marvig, Sommer, Jelsbak, Molin, & Johansen, 2015; E. E. Smith *et al.*,
1212006; Wilder *et al.*, 2009). The variant isolates are believed to elicit a strong flagella-driven
122immune response, which could play an important role in the establishment of biofilm-
123associated infections (LaFayette *et al.*, 2015).

124We compared PA14 WT with its *lasR* mutant in this study, in order to evaluate i)
125*P. aeruginosa* pathogenicity traits that are independent of the activity of *lasR* and ii) bacterial
126factors that contribute to the selective advantage of *lasR* mutants in chronic biofilm-associated
127infections.

128As *in vivo* model, we first employed acute infections of lungs by *P. aeruginosa* via a non-
129invasive installation of bacteria into the trachea of mice. Since the input Tn pool needed to
130contain the complex near-saturated Tn mutant library, the inoculum was carefully titrated to
131an optimal infection dose of 5×10^6 colony forming units (cfu) in 50 μ l. At this infection dose,
132highly reproducible bacterial colonization of the murine lung was achieved 24 post infection
133(pi) (Fig. S1). At 48 h and 72 h pi, indication of fast clearance of the bacterial colonization
134was observed, accompanied by a rapid body weight regain (Fig. S1). Histology of the
135*P. aeruginosa*-challenged lungs showed hemorrhaging, vacuolization and perivascular and
136alveolar inflammatory cell infiltration 24 h pi, whereas PBS-treated controls exhibited no
137pathological changes (Fig. 1).

138As second model we employed a transplantable tumor infection model to study biofilm-
139associated infections. This model had been established and intensively characterized
140previously by us (Crull *et al.*, 2011). Such subcutaneous tumor tissue is quickly colonized by
141the bacteria after intravenously infection of the mice. Biofilms develop within 24 h and the
142bacteria exhibit an *in vivo* transcriptional profile that resembles the *in vivo* transcriptional
143profile of *P. aeruginosa* residing in the cystic fibrosis lung (Komor *et al.*, 2012; Pawar *et al.*,
1442015a). To ensure that the near saturated Tn mutant libraries were fully represented in the
145input mutant pool, we adapted this tumor infection model and applied an inoculum of 5×10^6
146bacteria intra-tumorally (*it*). Under these conditions, the tumors were colonized in a very
147similar fashion compared to systemically infected mice. However, the dissemination of
148bacteria to other organs was restricted (Fig. S2- S4). The preferred niche of *P. aeruginosa*
149within the tumor appears to be at the interface of viable and necrotic regions (Fig. S5, Fig. 2).

Abundance of Tn mutants in input and output pools of *P. aeruginosa* PA14 and Δ lasR Tn mutant libraries

We first sequenced independent input mutant pools (5×10^6 bacteria) of the two Tn mutant libraries. For the PA14 Tn mutant library, we confirmed the highly saturated homogenous distribution of transposon insertions at genomic loci that contain thymine-adenine (TA) sites across the PA14 chromosome (Skurnik, Roux, Aschard, *et al.*, 2013). The chromosome of PA14 contains 5,826 genes that harbor two or more TA sites within the first 90 % of the gene sequence. For 5,188 genes, Tn insertions were detected in at least one of three independent input sequencing approaches. 82.8 % of those genes were found to be represented in all three input pools (Fig. S6). Similarly, analysis of 5×10^6 bacteria aliquots of input samples of the Δ lasR Tn mutant library also revealed a homogenous distribution of transposon insertions across the genome. 5,111 genes with at least two disrupted TA sites in the first 90 % of their sequence were detected in at least one out of five sequencing approaches. 88.6 % of those genes were identified in all five input mutant pools (Fig. S7).

To identify genes that influence *P. aeruginosa* pathogenicity during acute infections, we infected mice intra-tracheally with 5×10^6 bacteria of the PA14 Tn mutant library and the Δ lasR Tn mutant library (Fig. 3). After 24 h, the Tn mutant output pools were recovered by streaking lung homogenates onto agar plates. We also infected tumor-bearing mice intra-tumorally with 5×10^6 bacteria of the PA14 mutant library and the Δ lasR Tn mutant library. Tumor-passaged Tn mutants were recovered from homogenates 48 h pi on agar plates.

Subsequently, we subjected the *ex vivo* output Tn mutant pools to high-throughput sequencing. Overall, compared to the input sample the number of individual Tn mutants within the pools (and thus the diversity of the mutant pool) decreased in the *ex vivo* samples, indicating that a strong selection took place. The number of Tn tagged genes that were

175recovered in the output mutant pools of PA14 and $\Delta lasR$ following acute lung infections were
176overall 4,130 and 3,759, respectively (corresponding to 80 % and 65 % of the input mutant
177pools). Similarly, the number of tagged genes that were recovered following infection of
178tumors was 3,565 for PA14 and 1,510 for the $\Delta lasR$ mutants, respectively. This corresponds
179to 69 % and 26 % of the respective input mutant pools.

180**Identification of positively and negatively selected Tn mutants of distinct functional** 181**classes during acute infections**

182Analysis of the output pool from acute infections revealed positively and negatively selected
183Tn mutants. They were ranked by their fold-change (input/output) and false discovery rate
184(FDR) (S1 Table). Fig. 4 depicts the functional categories which were significantly
185overrepresented within the top 500 of the Tn-disrupted genes of the positively and negatively
186selected mutants in the acute infection model of both libraries. Table 1 lists the corresponding
187genes that were affected. From these lists it becomes clear that negative selection was mainly
188observed for genes involved in respiration and oxidative phosphorylation, indicating that
189these genes are functionally required. Positively selected mutants harbored Tn insertions in
190genes involved mainly in biogenesis and regulation of type IV pili and flagella, suggesting
191that *P. aeruginosa* isolates that lost these structures have a selective advantage.

192Obviously, mutants with Tn insertions within genes encoding different sub-units of the
193proton-pumping NADH:ubiquinone oxidoreductase, respiratory complex I, (*nuo* genes)
194accounted for the bulk of negatively selected mutants in the lungs during acute infections
195(Fig. 5). The NADH:ubiquinone oxidoreductase couples the electron transfer from NADH to
196ubiquinone with the translocation of protons across the membrane. This function is obviously
197of importance for the establishment of acute infections in both *P. aeruginosa* strains, the
198PA14 wildtype and the *lasR* mutant. On the other hand, mutants that lack type IV pili due to
199Tn insertions in genes encoding for structural components as well as type IV biogenesis (*pilA*,

200*B, E, M, N, O, P, Z, F, Q* and *fimU*) were positively selected upon passage of both, the PA14
 201and (to a lower extend) the Δ *lasR* mutant libraries, in the mouse lung (Fig. 6). This is
 202suggesting that bacteria lacking type IV pili are preferentially able to colonize the lung. This
 203is in agreement with the notion that the pilus structures are believed to be potential targets for
 204early immune recognition by host defense mechanisms. This would promote clearance of
 205*P. aeruginosa* (Lindestam Arlehamn & Evans, 2011; Sutterwala *et al.*, 2007). Indeed, a
 206positive association between lack of type IV pili and *P. aeruginosa* colonization has also been
 207reported in a previous study of *in vivo* determinants for mouse gastrointestinal tract
 208colonization and subsequent systemic dissemination (Skurnik, Roux, Aschard, *et al.*, 2013).

209In addition to type IV pili, the flagellar system of *P. aeruginosa* is known to activate the
 210immune system of the host (Andersen-Nissen *et al.*, 2005; Hayashi *et al.*, 2001; K. D. Smith
 211& Ozinsky, 2002). Strains with Tn insertions in several genes encoding flagella components
 212have been demonstrated to be positively selected during colonization of the gastrointestinal
 213(Skurnik, Roux, Aschard, *et al.*, 2013) and respiratory (Roux, Danilchanka, Guillard *et al.*,
 2142015) tract of mice. This suggests that it is beneficial for *P. aeruginosa* to avoid activation of
 215the host immunity via Toll-like Receptor 5 as it would promote bacterial clearance. In
 216accordance, in *P. aeruginosa* PA14 Tn mutants within 24 genes with inactivating insertions in
 217structural or flagellar biogenesis function genes (*flgG, B, C, F, H, I, C, J, E, L* and *fliJ, I, C,*
 218*O, D, F, Q, R* and *motB, C, D,* and *flhA, B*) were enriched in the acute murine infection model.
 219However, it was striking to see that none of those 24 genes were positively selected for in the
 220 Δ *lasR* mutant strain (Fig. 7).

221Identification of positively and negatively selected Tn mutants of distinct functional 222classes during biofilm-associated tumor infections

223We also ranked the positively or negatively selected Tn insertion mutants derived from the
 224biofilm-associated tumor infection by the fold-change (input/output) and false discovery rate

225(FDR) (S1 Table). We selected the top 500 Tn mutant candidates and assigned the transposon
226disrupted genes to their functional category. Table 2 lists the corresponding genes that were
227affected. Upon passage of the Tn mutant libraries in biofilm associated infections, mainly
228negative selection was observed. Interestingly, negative selection was observed almost
229exclusively in Tn mutants that harbored insertions in genes involved in respiration and
230oxidative phosphorylation. As observed already for the *in vivo* lung passage, mutants with Tn
231insertions within the *nuo* genes encoding sub-units of the proton-pumping NADH:ubiquinone
232oxidoreductase were negatively selected in the tumor tissue in both *P. aeruginosa* strains, the
233PA14 wildtype and the *lasR* mutant (Fig. 5). This indicates that a functional respiratory
234complex I is important for *in vivo* survival of *P. aeruginosa*.

235Opposed to the acute lung infection model, in the tumor model no functional categories were
236significantly overrepresented within the Tn-disrupted genes of the positively selected mutants
237of both libraries (Fig. 8). Thus, in stark contrast to the passage in the mouse lung, we did not
238find a positive selection of flagella negative mutants in the tumor model.

239

240Flagellin is required for the fitness of the $\Delta lasR$ mutant in the biofilm-associated tumor 241infection

242One of the most striking finding of our Tn-seq studies was that in contrast to the acute
243pneumonia infection, no inactivating mutants of flagella mutants of PA14 or $\Delta lasR$ were
244enriched in biofilm-associated infections. Apparently, it is advantageous for *P. aeruginosa* to
245express flagella during the chronic infection process. Since the $\Delta lasR$ mutant exhibited a
246slight loss of mutants with transposon insertions in the flagellar filament gene *fliC* (Fig 9 A),
247we generated a clean $\Delta lasR \Delta fliC$ double deletion mutant to corroborate the importance of
248flagella in chronic infections. We infected tumor-bearing mice with a mix of PA14 and the
249 $\Delta lasR$ mutant or of PA14 and the $\Delta lasR \Delta fliC$ double mutant (Fig. 9 B). No fitness advantage

250of the $\Delta lasR$ mutant over PA14 was observed. However, when both *lasR* and *fliC* were
251inactivated, a clear trend was observable. In most tumors, the $\Delta lasR \Delta fliC$ double mutant was
252outcompeted by the wildtype. Thus, flagellin appears to be an important factor also for
253survival of the $\Delta lasR$ mutant during chronic biofilm-associated infections.

254Discussion

255Some highly pathogenic microorganisms pursue the strategy to express a few but very
256effective virulence factors (Chandrasekaran & Lacy, 2017; Fowler *et al.*, 2017; M.-S. Lee,
257Koo, Jeong, & Tesh, 2016). In contrast, the success of the opportunistic pathogen
258*P. aeruginosa* can be attributed to a highly complex virulence-regulatory network
259(Balasubramanian, Schneper, Kumari, & Mathee, 2013). *P. aeruginosa* has evolved to
260express a fine-tuned broad array of virulence genes to enable infection and efficient evasion
261of host defense mechanisms (D. G. Lee *et al.*, 2006).

262To shed more light on the pathogenic landscape of *P. aeruginosa*, we investigated depletion
263or enrichment of transposon insertion mutants after *in vivo* selection in acute murine
264pneumonia or biofilm-associated murine tumor infection models. The Tn mutant library of
265*P. aeruginosa* PA14 had been used previously in a mouse infection model that mimics
266intestinal colonization and systemic dissemination of *P. aeruginosa* in humans (Skurnik,
267Roux, Aschard, *et al.*, 2013). While the previous study identified almost all known virulence
268factors to be of importance for PA14 *in vivo* dissemination, this was not the case in the acute
269pneumonia or the biofilm-associated tumor infection used in our study. Nevertheless, a
270common requirement was observed for genes involved in respiration, energy utilization and
271nutrition during both infection processes as indicated by the counter-selection of mutants
272bearing Tn insertions in such genes. Thus, especially respiration and oxidative
273phosphorylation seems to represent a metabolic phenotype of *P. aeruginosa* required for
274survival during the infection process in the mammalian host.

275 More recently, transposon sequencing technology has been used to identify genes important
276 for *P. aeruginosa* survival in acute burn and chronic surgical wound infections (Turner *et al.*,
277 2014). In accordance with our results, it was demonstrated that bacterial adaptation to
278 conditions encountered during such infection processes is accompanied by metabolic changes.
279 Similarly, only limited infection type-specific genetic pathways appeared to be important for
280 acute versus chronic courses of soft tissue infections. This is remarkable. It suggests that the
281 host response against the infectious agent rather than the actual use of specific virulence
282 factors by *P. aeruginosa* is the main driver for pathogenicity and duration of the disease
283 under distinct environmental conditions (Turner *et al.*, 2014).

284 The *P. aeruginosa* flagellar system is known to activate the host immune system via the toll-
285 like receptor five (TLR5) (Miao, Andersen-Nissen, Warren, & Aderem, 2007). In our acute
286 lung infections using the PA14 Tn mutant library a strong positive selection of mutations
287 inactivating flagella was observed. This indicates that the loss of the TLR5 stimulatory ligand
288 was supporting bacterial survival. Similarly, strains with inactivating insertions in several
289 genes encoding flagella components have been previously demonstrated to be positively
290 selected during colonization of the gastrointestinal tract in mice (Skurnik, Roux, Aschard,
291 *et al.*, 2013) and for acute lung infections (Roux *et al.*, 2015). Thus, a loss or lack of flagella
292 expression is advantageous for *P. aeruginosa* to avoid activation of the host immunity and to
293 prevent clearance from the infected site. In accordance, it was recently shown that, probably
294 due to the efficient recognition by the immune system, *P. aeruginosa* strains which produce
295 functional flagellin are more rapidly cleared in acute infection models (Lovewell *et al.*, 2011;
296 Patankar *et al.*, 2013; Ramphal *et al.*, 2008). This appeared to be independent of whether the
297 bacteria were motile or only produced flagellin (Balloy *et al.*, 2007).

298 However, high production of flagellin induced increased mortality rates in acute infection
299 models (Balloy *et al.*, 2007). Importantly, in the present study we found no enrichment of

300inactivating flagella mutants in the *lasR* mutant output pool neither in the acute nor biofilm-
301associated infection model. Thus, one could argue that in a $\Delta lasR$ mutant that lacks the
302production of an array of virulence factors, the presence of high amounts of flagellin might be
303important for the establishment of an acute pneumonia infection. A hyper-inflammatory
304response might efficiently substitute for the lack of other virulence factors (LaFayette *et al.*,
3052015). Along the same line, the type II secretion system (T2SS) is a target of LasR and it has
306been shown that the importance of the T2SS for bacterial pathogenicity can only be evaluated
307in a *fliC* mutant background (Jyot *et al.*, 2011).

308In contrast to the acute lung infection, during biofilm-associated infections, we did not
309observe a selective advantage for mutants that have lost their flagella. This implies that while
310during acute pneumonia infections it might be useful for *P. aeruginosa* to hide from the
311immune system. This is not the case in biofilm-associated infections. One explanation might
312be that the microenvironment of the tumor tissue down-regulates genes encoding flagella
313biosynthetic components. Thus, no selective advantage of respective mutant isolates would be
314encountered. On the other hand, the presence of flagella might aid the establishment of
315biofilms as flagella constitute a component of the extracellular biofilm matrix (Klausen *et al.*,
3162003). However, we did not observe any other component of the biofilm matrix to be selected
317against in the tumor model. A third explanation for the importance of flagella during biofilm-
318associated infections could be that flagellin elicits a host immune response and that this
319immune response is the key driver for *in vivo* biofilm formation as has been suggested before
320(Jensen, Givskov, Bjarnsholt, & Moser, 2010).

321Interestingly, in the $\Delta lasR$ Tn mutant library, mutants inactivating *fliC* were slightly
322underrepresented in the tumor infection. These results indicate that the presence of flagella
323might be advantageous. LasR governs the production of the two proteases AprA and LasB.
324Both have previously been demonstrated to degrade flagellin (Bardoel *et al.*, 2011; Casilag

325 *et al.*, 2015). Thus, it is conceivable that in tumors infected by the *lasR* mutant even more
326 flagellin is present than in PA14 colonized tumors.

327 In our biofilm-associated tumor infection model, the $\Delta lasR$ mutant did not out-compete PA14.
328 However, in accordance with a role of flagellin in the establishment or maintenance of
329 biofilm-associated tumor infections a clean $\Delta lasR \Delta fliC$ double deletion mutant was confirmed
330 to be weaker when competing with PA14 compared to the $\Delta lasR$ mutant. These results thus
331 corroborate the hypothesis that a flagellin-mediated induction of an inflammatory response
332 could be the main reason for the selection of *lasR* mutants in chronically infected CF lungs
333 (LaFayette *et al.*, 2015).

334 Taken together, our results indicate that adaptation of *P. aeruginosa* to the challenging
335 conditions of the human host as simulated by our mouse infection models requires the activity
336 of genes involved in respiration, energy utilization and nutrition during the infection
337 processes. Remarkably, most of those changes have been found independent of differences in
338 infection trajectories. Acuteness and biofilm formation could be rather dictated by the
339 immune response of the host against the bacteria. Our results thus highlight the complex
340 interplay of adaptive bacterial behavior and suggest a role of the immune response in
341 modulating severity and infection types.

342 **Experimental Procedures**

343 **Bacterial strains, cell lines and primers**

344 All bacterial strains used in this study are listed in Table S2. The donor strain for Tn
345 mutagenesis of the $\Delta lasR$ Tn mutant library, *E. coli* SM10 λ pir, carried the plasmid pBT20
346 encoding the mariner HimarI C9 transposon. Inoculums (input samples) of transposon mutant
347 libraries were prepared by plating bacteria from glycerol stocks on 20 LB agar plates, and
348 incubating them overnight at 37°C. Grown cells were pooled, washed and adjusted to a
349 infection dose of 5×10⁶cfus/50 μ l. CT26 colon carcinoma cells (ATCC CRL-2638) were
350 cultivated in monolayers in Iscove's modified Dulbecco's medium (IMDM) (Life
351 Technologies, Germany) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum
352 (Integro) and 250 mmol liter⁻¹ β -mercaptoethanol (Serva).

353 ***P. aeruginosa* $\Delta lasR$ Tn mutagenesis**

354 Tn mutagenesis was carried out by mating the $\Delta lasR$ deletion mutant with *E. coli* SM10 λ pir
355 [pBT20], followed by selection for *P. aeruginosa* cells harboring the Tn gentamicin-
356 resistance on gentamicin agar plates. In brief: donor *E. coli* cells (SM10 λ pir/pBT20) were
357 cultivated overnight at 37°C and *P. aeruginosa* was grown at 43°C overnight in LB. Cultures
358 were centrifuged, re-suspended, mixed at a 1:1 ratio before incubation for 2 h at 37°C. Mating
359 mixtures were then plated on Vogel-Bonner-Minimal (VBM) agar supplemented with
360 gentamicin (60 μ g/ml) and grown overnight at 37°C. $\Delta lasR$ Tn mutants were harvested,
361 glycerol stocks prepared and stored at minus 80°C.

362 **$\Delta lasR$ Tn mutant library sequencing library preparation**

363 Sequencing library preparation was performed as previously described for transposon
364 insertion directed sequencing (TraDIS) by Barquist *et al.* (Barquist *et al.*, 2016). In brief: 1
365 μ g DNA of the $\Delta lasR$ transposon mutant pools was sheared to approx. 300 bp fragments

366(Adaptive Focused Acoustics (AFA) technology, Covaris®, 10 % Duty cycle, 10%;
367Intensity 5; 200 Cycles and Time 60 s). The fragments were subsequently end-repaired (New
368England Biolabs Next End Repair Module) and dA-tailed (New England Biolabs Klenow). A
369splinkerette adapter was ligated to DNA fragments using TA ligase mastermix, according to
370the manufacturer's instructions (NEB Next Quick Ligation Module, NEB). Size selection
371(300 – 400 bp) and purification was performed using the Agencourt AMPure® XP-Kit (NEB,
372Beckman Coulter Life Sciences), following manufacturer's instructions. Next, fragments were
373amplified using SplAP5.x primers (8 nt barcode sequence) and High Fidelity Mastermix
374(NEBNext® High-Fidelity 2X PCR Master Mix). Quality was controlled by Bioanalyzer
375(Agilent genomics) and the library was subsequently subjected to Illumina sequencing using
376HiSeq 2500 in single-end mode (50 nt) with customized Read1primer and iPCRtagSeq
377primers. Primers are listed in Table S3.

378PA14 Tn mutant library preparation for sequencing

379Sequencing library preparation of the PA14 Tn mutant library was performed as published by
380Surnik *et al.* (Skurnik, Roux, Aschard, *et al.*, 2013). Briefly: 10 µg DNA of the PA14 Tn
381mutant pools were MmeI (NEB) digested and approx. 1,5 kb fragments were excised from 0.8
382% agarose gels. LIB_adaptBT adapters (with barcode) were ligated with T4 DNA ligase
383(NEB) overnight and gel-purified. Fragment amplification was carried out with LIB_PCR_5/3
384primers and Phusion® DNA polymerase (High Fidelity DNA polymerase, NEB). Sequencing
385library quality was verified by Bioanalyzer (Agilent genomics) and library fragments were
386sequenced on HiSeq 2500 in single-end mode (50 nt) with standard NEB Next primers.

387Animals

388Female 6-7 weeks old female BALB/c mice were purchased from Janvier (BALB/cByJRj).

389**Histology**

390 Mouse lungs and tumors were fixed for 24 h in 4 % buffered formaldehyde and embedded in
391 paraffin. Sections 3 μ m in thickness were prepared and stained with hematoxylin and eosin.
392 For immunostainings, a biotin coupled *P. aeruginosa* antibody was used at a 1:150 dilution
393 (polyclonal rabbit isotype IgG antibody, Biotrend). Following the addition of Streptavidin
394 conjugated with horseradish peroxidase the sections were incubated with 3,3'-
395 diaminobenzidine (DAB, Sigma). Analysis was carried out with an Olympus BX51
396 microscope with Olympus U-CMAD3 camera using the software ZEN 2009.

397**Live Imaging (IVIS®)**

398 Infection kinetics of PAO1/pCTX::lux infected tumor-bearing mice were visualized with the
399 IVIS® Spectrum *in vivo* imaging system (Xenogen IVIS® 200, Perkin Elmer, USA), following
400 the instructions of the manufacturer and analyzed using the Living image 3.0 software (Perkin
401 Elmer, USA).

402**Infection of tumor-bearing mice**

403 Mice were subcutaneously (*sc*) inoculated at the abdomen with 5×10^5 CT26 cells and tumors
404 were grown to a diameter of approx. 7 mm. Tumor-bearing mice were anesthetized by
405 inhalation of isofuran gas (2 % of IsoFlo®, Abbott) and intra-tumorally infected with 5×10^6
406 cfu of *P. aeruginosa* transposon library pools.

407**Lung infection**

408 Mice were anesthetized by intraperitoneal injection of 100 μ l/10 g bodyweight
409 ketamine/xylamine and infected non-invasively via the trachea by using an Introcan Safety
410 24G catheter (BI Braun), covered with a polyethylene tubing (Becton Dickinson). Bacterial
411 suspension of *P. aeruginosa* Tn mutant library pools (5×10^6 cfu) in 50 μ l PBS was
412 administered.

413CFU counts of infected tissue and recovery of *ex vivo* transposon mutants for sequencing

414Lung infected mice were sacrificed 24 h pi and tumor-infected mice 48 h pi. For cfu counts,
415organs were homogenized and plated in serial dilutions. For transposon sequencing, whole
416homogenates were plated, because the total number of transposon mutants in the tissue was
417too small for direct isolation of genomic bacterial DNA. Plating of the homogenate was done
418on overall 20 agar plates/animal in order to expand all mutants present and incubated
419overnight at 37°C. Bacterial material was scraped off, pooled per organ, centrifuged and
420genomic DNA extracted.

422Ethics statement

423All animal experiments performed were in agreement with the German Animal Welfare Act
424(Tierschutzgesetz, 1998) and international laws and policies governing the use of animals for
425scientific purposes (EC Council Directive 86/609, OJ L 358,1, 12 December 1987; and NIH
426Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).
427The protocol met the approval by the ethical board of the local authorities (Niedersächsisches
428Landesamt für Verbraucherschutz und Lebensmittelsicherheit [LAVES], Oldenburg,
429Germany). The permission number is 33.9.42502-04-050/09.

430Data analysis

431Sequence reads of the $\Delta lasR$ Tn mutant library were mapped to the reference strain
432*Pseudomonas aeruginosa* PA14 using bowtie2 (default settings) (Langmead & Salzberg,
4332012). TA sites were not part of the sequence reads and were therefore extracted according to
434the reference sequence. For the PA14 Tn mutant library, no mismatches were allowed because
435of their short genomic sequence length of 16-17 nt and TA sites were extracted from the 3'
436end of these sequences. Read counts had been duplicated for TA sites that were shared by two
437opposite genes.

438 To reduce the number of genes that are disrupted but still functional (false positives), we did
439 not consider mutants with transposon insertions within 10% from the 3' end of the gene. This
440 assumption is based on our observation that transposon-insertion mutants survive if essential
441 genes are disrupted in this region. Therefore, genes (with at least 10 TA sites) were split into
442 7 regions, three 5% regions at each end of the gene and 70% of the gene center. While there
443 was no allowed TA disruption in the gene center (assumed as essential genes), there had to be
444 at least one disruption at any other region of the gene. There was a clear trend that transposon
445 mutants with gene disruptions at the 5' end and 10-15% at the 3' end did not survive, but
446 those with disruptions at the 10% of the 3' end did (data not shown).

447 Disrupted genes were counted to allow direct comparison of different samples. Therefore, TA
448 reads were normalized using the weighted trimmed mean of M-values (TMM). To reduce
449 number of false positives, genes with at least 2 TA sites with $TMM > 1$ were defined as
450 disrupted genes.

451 Genes with at least 2 counts per million (cpm) in at least 4 samples were included in
452 functional enrichment analysis. Gene depletion and enrichment was calculated using the R
453 package edgeR (Robinson *et al.*, 2010). Because the size of the input mutant pool was
454 restricted in the mouse experiments, only few genes, which were depleted/enriched in the
455 output mutant pool passed the significance filter. Therefore, we focused on significant
456 functional enrichment of gene subsets within those pools, which exhibited an at least two-fold
457 enrichment/depletion. The analysis was performed on the top 500 genes, ordered according to
458 their p value. Functions (biological processes, Gene Ontology (Ashburner *et al.*, 2000;
459 The Gene Ontology Consortium, 2017) of these genes were defined to be significantly
460 enriched using the hypergeometric test (R function phyper, FDR adjusted p value ≤ 0.05).

461 Raw transposon sequencing data have been submitted to the European Nucleotide Archive
462 (ENA) under the accession number PRJEB26817.

464 Acknowledgement

465 This work was supported by an ERC Consolidator Grant (COMBAT 724290) and by the
 466 German Research Foundation (DFG) via a scholarship given to AL in the frame of the
 467 International Research and Training Group (IRTG, Speaker S. Suerbaum, Grant Number:
 468 1273. VP was supported by INNO INDIGO Partnership Program (INDIGO-IPP2-060).
 469 The funders had no role in study design, data collection and analysis, decision to publish, or
 470 preparation of the manuscript.

471

472 **Conflict of interest** The authors declare that they have no conflict of interest.

473

474 References

475 Andersen-Nissen, E., Smith, K. D., Strobe, K. L., Barrett, S. L. R., Cookson, B. T., Logan, S. M., &
 476 Aderem, A. (2005). Evasion of Toll-like receptor 5 by flagellated bacteria. *Proceedings of the*
 477 *National Academy of Sciences*, 102, 9247–9252.

478 Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., ... Sherlock, G.
 479 (2000). Gene Ontology: tool for the unification of biology. *Nature Genetics*, 25, 25–29.

480 Balasubramanian, D., Schneper, L., Kumari, H., & Mathee, K. (2013). A dynamic and intricate
 481 regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Research*, 41,
 482 1–20.

483 Balloy, V., Verma, A., Kuravi, S., Si-Tahar, M., Chignard, M., & Ramphal, R. (2007). The Role of
 484 Flagellin versus Motility in Acute Lung Disease Caused by *Pseudomonas aeruginosa*. *The*
 485 *Journal of Infectious Diseases*, 196, 289–296.

486Bardoel, B. W., van der Ent, S., Pel, M. J. C., Tommassen, J., Pieterse, C. M. J., van Kessel, K. P. M.,
487 & van Strijp, J. A. G. (2011). *Pseudomonas* evades immune recognition of flagellin in both
488 mammals and plants. *PLoS Pathogens*, 7, e1002206.

489Barquist, L., Mayho, M., Cummins, C., Cain, A. K., Boinett, C., Page, A. J., ... Parkhill, J. (2016).
490 The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries.
491 *Bioinformatics (Oxford, England)*. doi:10.1093/bioinformatics/btw022

492Bianconi, I., Milani, A., Cigana, C., Paroni, M., Levesque, R. C., Bertoni, G., & Bragonzi, A. (2011).
493 Positive signature-tagged mutagenesis in *Pseudomonas aeruginosa*: tracking patho-adaptive
494 mutations promoting airways chronic infection. *PLoS Pathogens*, 7, e1001270.

495Bielecki, P., Puchałka, J., Wos-Oxley, M. L., Loessner, H., Glik, J., Kawecki, M., ... dos Santos, V.
496 A. P. M. (2011). In-Vivo Expression Profiling of *Pseudomonas aeruginosa* Infections Reveals
497 Niche-Specific and Strain-Independent Transcriptional Programs. *PLoS ONE*, 6, e24235.

498Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. *APMIS. Supplementum*, 1–
499 51.

500Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, S. R., Moser, C., Kühl, M., ... Høiby, N.
501 (2013). The in vivo biofilm. *Trends in Microbiology*, 21, 466–474.

502Breidenstein, E. B. M., de la Fuente-Núñez, C., & Hancock, R. E. W. (2011). *Pseudomonas*
503 *aeruginosa*: all roads lead to resistance. *Trends in Microbiology*, 19, 419–426.

504Cabrol, S., Olliver, A., Pier, G. B., Andremont, A., & Ruimy, R. (2003). Transcription of quorum-
505 sensing system genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Journal*
506 *of Bacteriology*, 185, 7222–30.

507Casilag, F., Lorenz, A., Krueger, J., Klawonn, F., Weiss, S., & Häussler, S. (2015). The LasB Elastase
508 of *Pseudomonas aeruginosa* Acts in Concert with Alkaline Protease AprA To Prevent Flagellin-
509 Mediated Immune Recognition. *Infection and Immunity*, 84, 162–171.

510Chandrasekaran, R., & Lacy, D. B. (2017). The role of toxins in *Clostridium difficile* infection. *FEMS*
43
44

511 *Microbiology Reviews*, 41, 723–750.

512 Chatzinikolaou, I., Abi-Said, D., Bodey, G. P., Rolston, K. V. I., Tarrand, J. J., & Samonis, G. (2000).

513 Recent Experience With *Pseudomonas aeruginosa* Bacteremia in Patients With Cancer. *Archives*

514 *of Internal Medicine*, 160, 501.

515 Crouch Brewer, S., Wunderink, R. G., Jones, C. B., & Leeper, K. V. (1996). Ventilator-associated

516 pneumonia due to *Pseudomonas aeruginosa*. *Chest*, 109, 1019–29.

517 Crull, K., Rohde, M., Westphal, K., Loessner, H., Wolf, K., Felipe-López, A., ... Weiss, S. (2011).

518 Biofilm formation by *Salmonella enterica* serovar Typhimurium colonizing solid tumours.

519 *Cellular Microbiology*, 13, 1223–1233.

520 D’Argenio, D. A., Wu, M., Hoffman, L. R., Kulasekara, H. D., Déziel, E., Smith, E. E., ... Miller, S. I.

521 (2007). Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of

522 cystic fibrosis patients. *Molecular Microbiology*, 64, 512–533.

523 Dubern, J.-F., Cigana, C., De Simone, M., Lazenby, J., Juhas, M., Schwager, S., ... Cámara, M.

524 (2015). Integrated whole-genome screening for *Pseudomonas aeruginosa* virulence genes using

525 multiple disease models reveals that pathogenicity is host specific. *Environmental Microbiology*,

526 17, 4379–4393.

527 Feltner, J. B., Wolter, D. J., Pope, C. E., Groleau, M.-C., Smalley, N. E., Greenberg, E. P., ...

528 Dandekar, A. A. (2016). LasR Variant Cystic Fibrosis Isolates Reveal an Adaptable Quorum-

529 Sensing Hierarchy in *Pseudomonas aeruginosa*. *MBio*, 7. doi:10.1128/mBio.01513-16

530 Folkesson, A., Jelsbak, L., Yang, L., Johansen, H. K., Ciofu, O., Høiby, N., & Molin, S. (2012).

531 Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective.

532 *Nature Reviews Microbiology*, 10, 841–851.

533 Fowler, C. C., Chang, S.-J., Gao, X., Geiger, T., Stack, G., & Galán, J. E. (2017). Emerging insights

534 into the biology of typhoid toxin. *Current Opinion in Microbiology*, 35, 70–77.

535 Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., & Lory, S. (2004). A Signaling

536 Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence
537 in *Pseudomonas aeruginosa*. *Developmental Cell*, 7, 745–754.

538 Hancock, R. E., Mutharia, L. M., Chan, L., Darveau, R. P., Speert, D. P., & Pier, G. B. (1983).
539 *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive,
540 nontypable strains deficient in lipopolysaccharide O side chains. *Infection and Immunity*, 42,
541 170–7.

542 Häussler, S., Tümmler, B., Weissbrodt, H., Rohde, M., & Steinmetz, I. (1999). Small-colony variants
543 of *Pseudomonas aeruginosa* in cystic fibrosis. *Clinical Infectious Diseases: An Official*
544 *Publication of the Infectious Diseases Society of America*, 29, 621–5.

545 Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., ... Aderem, A. (2001).
546 The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*,
547 410, 1099–1103.

548 Jensen, P. Ø., Givskov, M., Bjarnsholt, T., & Moser, C. (2010). The immune system vs. *Pseudomonas*
549 *aeruginosa* biofilms. *FEMS Immunology & Medical Microbiology*, 59, no-no.

550 Jyot, J., Balloy, V., Jouvion, G., Verma, A., Touqui, L., Huerre, M., ... Ramphal, R. (2011). Type II
551 secretion system of *Pseudomonas aeruginosa*: in vivo evidence of a significant role in death due
552 to lung infection. *The Journal of Infectious Diseases*, 203, 1369–77.

553 Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., & Tolker-
554 Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type
555 IV pili mutants. *Molecular Microbiology*, 48, 1511–1524.

556 Klockgether, J., & Tümmler, B. (2017). Recent advances in understanding *Pseudomonas aeruginosa* as
557 a pathogen. *F1000Research*, 6, 1261.

558 Komor, U., Bielecki, P., Loessner, H., Rohde, M., Wolf, K., Westphal, K., ... Häussler, S. (2012).
559 Biofilm formation by *Pseudomonas aeruginosa* in solid murine tumors – a novel model system.
560 *Microbes and Infection*, 14, 951–958.

561Kwon, Y. M., Ricke, S. C., & Mandal, R. K. (2016). Transposon sequencing: methods and expanding
562 applications. *Applied Microbiology and Biotechnology*, 100, 31–43.

563LaFayette, S. L., Houle, D., Beaudoin, T., Wojewodka, G., Radzioch, D., Hoffman, L. R., ... Nguyen,
564 D. (2015). Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing lasR mutants cause
565 hyperinflammatory responses. *Science Advances*, 1. doi:10.1126/sciadv.1500199

566Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*,
567 9, 357–359.

568Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., ... Ausubel, F. M.
569 (2006). Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial.
570 *Genome Biology*, 7, R90.

571Lee, M.-S., Koo, S., Jeong, D., & Tesh, V. (2016). Shiga Toxins as Multi-Functional Proteins:
572 Induction of Host Cellular Stress Responses, Role in Pathogenesis and Therapeutic Applications.
573 *Toxins*, 8, 77.

574Lesprit, P., Faurisson, F., Join-Lambert, O., Roudot-Thoraval, F., Foglino, M., Vissuzaine, C., &
575 Carbon, C. (2003). Role of the Quorum-sensing System in Experimental Pneumonia due to
576 *Pseudomonas aeruginosa* in Rats. *American Journal of Respiratory and Critical Care Medicine*,
577 167, 1478–1482.

578Lindestam Arlehamn, C. S., & Evans, T. J. (2011). *Pseudomonas aeruginosa* pilin activates the
579 inflammasome. *Cellular Microbiology*, 13, 388–401.

580Lorè, N. I., Cigana, C., De Fino, I., Riva, C., Juhas, M., Schwager, S., ... Bragonzi, A. (2012). Cystic
581 Fibrosis-Niche Adaptation of *Pseudomonas aeruginosa* Reduces Virulence in Multiple Infection
582 Hosts. *PLoS ONE*, 7, e35648.

583Lovewell, R. R., Collins, R. M., Acker, J. L., O'Toole, G. A., Wargo, M. J., & Berwin, B. (2011).
584 Step-Wise Loss of Bacterial Flagellar Torsion Confers Progressive Phagocytic Evasion. *PLoS*
585 *Pathogens*, 7, e1002253.

586Luzar, M. A., & Montie, T. C. (1985). Avirulence and altered physiological properties of cystic
587 fibrosis strains of *Pseudomonas aeruginosa*. *Infection and Immunity*, 50, 572–6.

588Mahenthiralingam, E., Campbell, M. E., & Speert, D. P. (1994). Nonmotility and phagocytic
589 resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic
590 fibrosis. *Infection and Immunity*, 62, 596–605.

591Marvig, R. L., Sommer, L. M., Jelsbak, L., Molin, S., & Johansen, H. K. (2015). Evolutionary insight
592 from whole-genome sequencing of *Pseudomonas aeruginosa* from cystic fibrosis patients. *Future*
593 *Microbiology*, 10, 599–611.

594Marvig, R. L., Sommer, L. M., Molin, S., & Johansen, H. K. (2015). Convergent evolution and
595 adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nature Genetics*, 47,
596 57–64.

597McManus, A. T., Mason, A. D., McManus, W. F., & Pruitt, B. A. (1985). Twenty-five year review of
598 *Pseudomonas aeruginosa* bacteremia in a burn center. *European Journal of Clinical*
599 *Microbiology*, 4, 219–23.

600Miao, E. A., Andersen-Nissen, E., Warren, S. E., & Aderem, A. (2007). TLR5 and Ipaf: dual sensors
601 of bacterial flagellin in the innate immune system. *Seminars in Immunopathology*, 29, 275–288.

602Nathwani, D., Raman, G., Sulham, K., Gavaghan, M., & Menon, V. (2014). Clinical and economic
603 consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas aeruginosa*
604 infections: a systematic review and meta-analysis. *Antimicrobial Resistance and Infection*
605 *Control*, 3, 32.

606Oliver, A., Cantón, R., Campo, P., Baquero, F., & Blázquez, J. (2000). High frequency of
607 hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science (New York,*
608 *N.Y.)*, 288, 1251–4.

609Osmon, S., Ward, S., Fraser, V. J., & Kollef, M. H. (2004). Hospital mortality for patients with
610 bacteremia due to *Staphylococcus aureus* or *Pseudomonas aeruginosa*. *Chest*, 125, 607–16.

611 Patankar, Y. R., Lovewell, R. R., Poynter, M. E., Jyot, J., Kazmierczak, B. I., & Berwin, B. (2013).
612 Flagellar motility is a key determinant of the magnitude of the inflammasome response to
613 *Pseudomonas aeruginosa*. *Infection and Immunity*, *81*, 2043–2052.

614 Pawar, V., Komor, U., Kasnitz, N., Bielecki, P., Pils, M. C., Gocht, B., ... Häussler, S. (2015a). In
615 Vivo Efficacy of Antimicrobials against Biofilm-Producing *Pseudomonas aeruginosa*.
616 *Antimicrobial Agents and Chemotherapy*, *59*, 4974–81.

617 Pawar, V., Komor, U., Kasnitz, N., Bielecki, P., Pils, M. C., Gocht, B., ... Häussler, S. (2015b). In
618 vivo efficacy of antimicrobials against biofilm producing *Pseudomonas aeruginosa*.
619 *Antimicrobial Agents and Chemotherapy*, *59*, AAC.00194-15.

620 Potvin, E., Lehoux, D. E., Kukavica-Ibrulj, I., Richard, K. L., Sanschagrin, F., Lau, G. W., &
621 Levesque, R. C. (2003). In vivo functional genomics of *Pseudomonas aeruginosa* for high-
622 throughput screening of new virulence factors and antibacterial targets. *Environmental*
623 *Microbiology*, *5*, 1294–308.

624 Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., ... Tompkins, R.
625 G. (2000). Plants and animals share functionally common bacterial virulence factors.
626 *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 8815–21.

627 Rahme, L. G., Stevens, E. J., Wolford, S. F., Shao, J., Tompkins, R. G., & Ausubel, F. M. (1995).
628 Common virulence factors for bacterial pathogenicity in plants and animals. *Science (New York,*
629 *N.Y.)*, *268*, 1899–902.

630 Ramphal, R., Balloy, V., Jyot, J., Verma, A., Si-Tahar, M., & Chignard, M. (2008). Control of
631 *Pseudomonas aeruginosa* in the lung requires the recognition of either lipopolysaccharide or
632 flagellin. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*, 586–92.

633 Robinson, M. D., McCarthy, D. J., Smyth, G. K., Moor, B. De, Brazma, A., Huber, W., ... Zhang, J.
634 (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene
635 expression data. *Bioinformatics*, *26*, 139–140.

636 Roux, D., Danilchanka, O., Guillard, T., Cattoir, V., Aschard, H., Fu, Y., ... Skurnik, D. (2015).
637 Fitness cost of antibiotic susceptibility during bacterial infection. *Science Translational*
638 *Medicine*, 7, 1–12.

639 Rumbaugh, K. P., Griswold, J. A., Iglewski, B. H., & Hamood, A. N. (1999). Contribution of quorum
640 sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infection and*
641 *Immunity*, 67, 5854–62.

642 Skurnik, D., Roux, D., Aschard, H., Cattoir, V., Yoder-Himes, D., Lory, S., & Pier, G. B. (2013). A
643 Comprehensive Analysis of In Vitro and In Vivo Genetic Fitness of *Pseudomonas aeruginosa*
644 Using High-Throughput Sequencing of Transposon Libraries. *PLoS Pathogens*, 9, e1003582.

645 Skurnik, D., Roux, D., Cattoir, V., Danilchanka, O., Lu, X., Yoder-Himes, D. R., ... Pier, G. B.
646 (2013). Enhanced in vivo fitness of carbapenem-resistant oprD mutants of *Pseudomonas*
647 *aeruginosa* revealed through high-throughput sequencing. *Proceedings of the National Academy*
648 *of Sciences of the United States of America*, 110, 20747–52.

649 Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D’Argenio, D. A., ...
650 Olson, M. V. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic
651 fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of*
652 *America*, 103, 8487–92.

653 Smith, K. D., & Ozinsky, A. (2002). Toll-like receptor-5 and the innate immune response to bacterial
654 flagellin. *Current Topics in Microbiology and Immunology*, 270, 93–108.

655 Sutterwala, F. S., Mijares, L. A., Li, L., Ogura, Y., Kazmierczak, B. I., & Flavell, R. A. (2007).
656 Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome.
657 *The Journal of Experimental Medicine*, 204, 3235–45.

658 Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G., & Ausubel, F. M. (1999). *Pseudomonas*
659 *aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors.
660 *Proceedings of the National Academy of Sciences of the United States of America*, 96, 2408–13.

661Tang, H. B., DiMango, E., Bryan, R., Gambello, M., Iglewski, B. H., Goldberg, J. B., & Prince, A.
662 (1996). Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of
663 pneumonia in a neonatal mouse model of infection. *Infection and Immunity*, 64, 37–43.

664The Gene Ontology Consortium. (2017). Expansion of the Gene Ontology knowledgebase and
665 resources. *Nucleic Acids Research*, 45, D331–D338.

666Turner, K. H., Everett, J., Trivedi, U., Rumbaugh, K. P., & Whiteley, M. (2014). Requirements for
667 *Pseudomonas aeruginosa* Acute Burn and Chronic Surgical Wound Infection. *PLoS Genetics*, 10,
668 e1004518.

669Wagner, V. E., & Iglewski, B. H. (2008). *P. aeruginosa* Biofilms in CF Infection. *Clinical Reviews in*
670 *Allergy & Immunology*, 35, 124–134.

671Wilder, C. N., Allada, G., & Schuster, M. (2009). Instantaneous Within-Patient Diversity of
672 *Pseudomonas aeruginosa* Quorum-Sensing Populations from Cystic Fibrosis Lung Infections.
673 *Infection and Immunity*, 77, 5631–5639.

674

675Tables

676Table 1 Functional enrichment of negatively and positively enriched transposon mutants in acute lung

677infection

Tn mutant bank	Enrichment	Functional classes	FDR value	All genes with Tn insertion from top 500 listGenes with Tn insertion
PA14 wt	Negative selection	Generation of precursor metabolites and energy	4.1×10^{-7}	<i>nuoN, nuoB, nuoD, nqrF, nuoH, nuoK, crc, nuoE, ubiH, ccmG, nuoG, trpE, cioB, nqrB, nqrC, nuoL, nqrD, nqrA, nuoF, nuoM, cycB, zwf, idh, ccoN, nuoI</i>
		Tryptophan biosynthetic process	0.0063	<i>trpG, trpB, trpE, trpC, aroQ2</i>
		Histidine biosynthetic process	0.0153	<i>hisE, hisI, hisD, hisH, hisC1, hisA</i>
		Oxidative phosphorylation	5.46×10^{-7}	<i>nuoB, nuoD, nuoH, nuoK, nuoE, nuoG, nuoL, nuoI</i>
	Positive selection	Type IV pilus-dependent motility	0.0002	<i>algR, pilO, pilP, pilE, pilN, fimU, pilB, pilA, pilM, pilY1, pilW, pilX</i>
		Type IV pilus-dependent biogenesis	1.46×10^{-6}	<i>pilO, pilP, pilE, pilN, fimU, pilB, pilD, pilQ, pilM, pilW</i>
		Pilus assembly	0.0100	<i>pilZ, pilE, fimU, pilB, pilD, pilQ, pilW, pilX</i>
		Bacterial-type flagellum-dependent cell motility	8.98×10^{-8}	<i>flgG, flgB, flgF, flgH, flgI, flgC, flgJ, fliJ, flgE, flgL, fliI, motD, motC, fliC, motB, fliF, PA14_69100</i>
		Bacterial-type flagellum assembly	7.26×10^{-12}	<i>flgG, flgB, flgF, flhB, flgI, flgC, flgJ, flhA, flg, flgL, fliI, fliQ, fliR</i>
		Bacterial-type flagellum organization	0.0012	<i>flgG, flgF, fliD, fliC, fliO</i>
$\Delta lasR$	Negative selection	Bacterial-type flagellum-dependent swimming motility	0.0406	<i>lon, ccmF, ccmC, PA14_66100</i>
		Generation of precursor metabolites and energy	0.0029	<i>exaB, glpD, crc, ccmG, nuoN, nqrD, nuoM, asp, ccmH, nuoG, nuoE, nuoK, nqrF, nuoL, nuoD</i>
		Cytochrome complex assembly	0.0025	<i>ccmB, ccmE, ccmF, PA14_45340, ccmH, ccmC</i>
		Oxidative phosphorylation	4.83×10^{-5}	<i>nuoG, nuoE, nuoK, nuoL, nuoD</i>
		Cellular response to antibiotics	0.0241	<i>clpP, lon, phoQ, typA, nuoN, ydhD, PA14_00120</i>
	Positive selection	Type IV pilus-dependent motility	6.17×10^{-4}	<i>pilE, fimU, algR, pilB, pilA, pilP, pilM, pilO, pilF, pilN</i>
		Type IV pilus-dependent biogenesis	4.60×10^{-6}	<i>pilE, fimU, pilB, pilP, pilQ, pilM, pilO, pilF, pilN</i>
		Pilus assembly	0.0255	<i>pilE, fimU, pilB, pilZ, pilQ, pilF</i>

678

679

680Table 2 Functional enrichment of negatively selected Tn mutants during biofilm-associated infections

Tn mutant bank	Functional class	FDR value	All genes with Tn insertion from top 500 list
PA14	Oxidative phosphorylation	1.00×10 ⁻⁶	<i>nuoL, nuoK, nuoI, nouH, nuoG, nuoE, nuoB, nuoA, nuoD</i>
	Generation of precursor metabolites and energy	0.0056	<i>nuoA, nuoB, nuoD, nuoE, nuoF, nuoG, nuoH, nuoI, nuoK, nuoL, nuoM, nuoN, narH, narI, nwrD, nqrF, pykA, crc, napA, aspP</i>
	Zinc II ion transport	0.0395	<i>znuA, znuC</i>
<i>ΔlasR</i>	Oxidative phosphorylation	0.0119	<i>nuoK, nuoJ, nuoI, nuoG, nuoE, nuoB</i>
	Cytochrome complex assembly	0.0223	<i>ccmH, ccmG, ccmF, ccmE, PA14_45340, ccmC</i>
	Cellular response to antibiotic	0.0120	<i>clpP, nuoN, phoQ, ydhD, mpl, phoP, typA, PA14_00120</i>

681

Fig 1. Histopathology of *P. aeruginosa* infected murine lungs

Lungs of BALB/c instilled with *P. aeruginosa* were removed 24 h pi, formalin fixated, embedded in Paraffin, sectioned at 3 μm thickness and stained with H & E. Control mice (A, B) were treated with PBS only. Mice (C, D) were infected with 5×10^6 cfu *P. aeruginosa*. (A, C) Overview of the right superior lobe at low magnification (scalebar 200 μm), (B, D) inset at higher magnification (scalebar 50 μm). (E) Infected lungs show areas with cytoplasmic vacuolization (thick black arrows) and innate immune cell infiltration (thin black arrows) (scalebar 20 μm).

691

692**Fig. 2. Immunohistology shows cluster formation of *P. aeruginosa* in murine CT26 tumors**

693Histological analysis of intra-tumorally or *intravenously* infected tumors (scalebar 100 μ m), stained
 694with H&E (A-C) or with an anti-*P. aeruginosa* antibody (D-F) counterstained with diaminobenzidine.
 695Arrows indicate presence of bacterial clusters.

Fig 3. Schematic experimental design. Two *Tn* mutant libraries (input) were applied to either a murine acute lung infection model or a murine biofilm-associated tumor infection model. Individual clones of *Tn* mutants are indicated by different colors and color intensity. After *in vivo* selection, clones of the output pools were recovered from tissue homogenates and subjected to transposon sequencing. Comparison of clonal frequencies in the input pool and the output pool revealed neutral or positive and negative selection of the mutants *in vivo*.

704

705Fig. 4. Functional enrichment of Tn mutants in acute lung infection

706Tn mutant pools were instilled into murine lungs and recovered from homogenates 24 h after
707instillation (infection dose of 5×10^6 cfu). The functional enrichment of mutants assorted to relevant
708biological processes is shown for depletion (left panel) and enrichment (right panel). The reference
709percentage (grey) was calculated with the whole *P. aeruginosa* genome. The functional enrichment of
710PA14 Tn mutants of one independent experiment (n=5) is shown by light pink bars. Enrichment of
711Δ*lasR* transposon mutants from two independent experiments (n = 7) is shown in light blue bars.
712Enrichment was calculated by preparing ranked top 500 lists of lung (output) vs inoculum (input) data.
713Terms of biological processes are defined by the Gene Ontology Consortium. Asterisks denote
714significant enrichment.

715

716

717**Fig 5. TA site sequencing reads in the nuo operon.**

718Members of the NADH dehydrogenase (*nuo*) gene family were jointly depleted in acute lung
719infections and biofilm-associated tumor infections in both transposon mutant libraries (PA14 wt and
720 Δ *lasR*). The bottom of the figure depicts the corresponding *nuo* genes (grey horizontal bars). TA sites
721present within these genes are indicated by black vertical bars. The left and the right panels show
722averaged and log transformed TMM-normalized TA reads for the transposon library of PA14 wt (two
723independent experiments) and Δ *lasR* (two independent experiments), respectively. Each panel depicts
724the results for acute lung infection (lung; PA14 wt *n* = 10, Δ *lasR* *n* = 7), biofilm-associated tumor
725infection (tumor; PA14 wt *n* = 10, Δ *lasR* *n* = 10) and the inoculum (inoc).

726

727**Fig 6 TA site sequencing reads in the pil operon**

728Members of the pilus (*pil*) gene family show increased transposon disruptions in acute lung infections
 729of the PA14 wt and $\Delta lasR$ transposon mutant library. For detailed description see figure 5.

730

731

732

733Fig. 7 TA site sequencing reads in the *flg* operon

734Members of the flagellum (*flg*) gene family that were jointly enriched in transposon disruptions in
 735acute lung infections of the PA14 wt but not the $\Delta lasR$ transposon mutant library. For detailed
 736description see figure 5.

737

738

739

740

741

742

743 Fig 8 Functional enrichment of Tn mutants in biofilm-associated tumor infections.

744 Transposon mutant pools were infected directly into the tumor tissue (infection dose: 5×10^6 cfu). After
 745 48 h mutant clones were recovered from tumor homogenates. The functional enrichment of mutants
 746 assorted to relevant biological processes is shown for depletion (left panel) and enrichment (right
 747 panel). The reference percentage (grey) was calculated with the whole *P. aeruginosa* genome. The
 748 functional enrichment of PA14 wt Tn mutants of one independent experiment ($n = 5$) is shown by dark
 749 red bars. Enrichment of $\Delta lasR$ transposon mutants from two independent experiments ($n = 10$) is
 750 shown in dark blue bars. Enrichment was calculated using ranked top 500 lists of lung (output) vs
 751 inoculum (input) data. Biological process terms are defined by the Gene Ontology Consortium.
 752 Asterisks denote significant enrichment.

753

754

755 **Fig. 9 In vivo fitness of *lasR/fliC* mutants.**

756 A) Tn insertion sites of the *fliC* gene. The lower part of the figure depicts all present TA sites within
 757 the first 90 % of the *fliC* gene (black vertical bars). Panels show the averaged TMM normalized TA
 758 reads for the $\Delta lasR$ transposon mutant bank (two independent experiments). The top and bottom
 759 micrographs show TA site hits for biofilm-associated tumor infections ($n = 10$) and the input sample,
 760 respectively. B) Competitive index (CI) of $\Delta lasR$ and $\Delta lasR \Delta fliC$ strains versus PA14. BALB/c mice
 761 were subcutaneously injected with 5×10^5 CT26 cells. After tumor development (about 10 days) mice
 762 were intravenously injected (5×10^6 cfu) with equal amounts of PA14 and $\Delta lasR$, or $\Delta lasR \Delta fliC$ mutant
 763 strains. Bacteria were recovered from the tumor tissue 48 hours post inoculation. CI values were
 764 calculated as the ratio of colonies recovered from mutant strain versus wild-type strain (output)
 765 divided by the ratio of mutant strain to wild-type strain present in the inoculum (input). The scatter
 766 plot displays values obtained for individual mice, and the mean and standard deviation are shown.
 767 Groups were tested non-significantly different by Mann-Whitney test ($p = 0.1685$). However, an
 768 obvious trend becomes apparent.

769