ARTICLE

Establishment of an induced memory response in *Pseudomonas aeruginosa* during infection of a eukaryotic host

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Abstract

In a given habitat, bacterial cells often experience recurrent exposures to the same environmental stimulus. The ability to memorize the past event and to adjust current behaviors can lead to efficient adaptation to the recurring stimulus. Here we demonstrate that the versatile bacterium *Pseudomonas aeruginosa* adopts a virulence phenotype after serial passage in the invertebrate model host *Galleria mellonella*. The virulence phenotype was not linked to the acquisition of genetic variations and was sustained for several generations, despite cultivation of the ex vivo virulence-adapted *P. aeruginosa* cells under rich medium conditions in vitro. Transcriptional reprogramming seemed to be induced by a host-specific food source, as reprogramming was also observed upon cultivation of *P. aeruginosa* in rich medium supplemented with polyunsaturated long-chain fatty acids. The establishment of induced memory responses adds a time dimension and seems to fill the gap between long-term evolutionary genotypic adaptation and short-term induced individual responses. Efforts to unravel the fundamental mechanisms that underlie the carry-over effect to induce such memory responses will continue to be of importance as hysteretic behavior can serve survival of bacterial populations in changing and challenging habitats.

Introduction

Pseudomonas aeruginosa is a versatile environmental bacterium and opportunistic human pathogen, which can thrive in a large variety of different and challenging habitats. The natural environment can serve as a reservoir

for *P. aeruginosa* to occasionally cause opportunistic infections in a wide range of hosts and tissues [1, 2]. Most of those host habitats are sub-optimal for sustained growth and thus provide strong selection for adaptive changes. Especially in the chronically infected cystic fibrosis lung, *P. aeruginosa* populations exist for a long-enough time for the positive selection of adaptive mutations to take place [3, 4]. Several studies have shown that there are a number of bacterial traits that show the signature of within-patient directional selection [5–7].

In addition to the evolution of adaptive mutations, phenotypic plasticity allows bacterial adaptation to a changing environment [8–11]. Although genetic diversity increases the potential of whole populations to adapt to changes in environmental conditions, a plastic phenotypic response to variations in the environment increases the fitness of the individual bacterium [12]. More recently, it has been argued that another response mechanism fills the gap between long-term evolutionary adaptation and short-term individual response [13]. This includes the incorporation of a time dimension and is characterized by the expression of a phenotypic response for several generations despite a change in the

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conditions that first induced the phenotypic response. This hysteretic behavior is also called response memory, as gene expression persists after removal of the external inducer [12, 14–16].

In this study we exposed a clinical *P. aeruginosa* isolate to the challenging environment within a eukaryotic host. We aimed to evaluate whether virulence could be induced upon host passages and which factors would be involved in the expression of virulence. Infections of caterpillar larvae have increasingly been used as a surrogate to study host–pathogen interactions in a range of microorganisms including *P. aeruginosa* [17–22]. We therefore passaged the *P. aeruginosa* clinical isolate multiple times in larvae of the wax moth *Galleria mellonella*. The immune system of the *G. mellonella* larvae shows functional similarities to human innate immune responses such as expression of antimicrobial peptides, lysozyme, generation of reactive oxygen species, and phagocytosis of invading microbes [23–26].

Genotypic and phenotypic analyses of the in vivo-passaged *P. aeruginosa* isolate uncovered extensive transcriptional reprogramming and phenotypic switching to higher virulence. This phenotypic switch could be in large parts recapitulated by growing the bacteria for several passages in rich medium with an excess of long-chain polyunsaturated fatty acids. The latter have been demonstrated to be enriched in the hemolymph of the insect larvae [27]. Importantly, the phenotypic changes were not associated with genotypic changes and were lost after cultivation of the in vivo and the in vitro evolved strain for several passages under rich medium culture conditions. Our results highlight that in addition to a non-inducible genotypic and a well-studied inducible plastic response, the opportunistic pathogen *P. aeruginosa* also produces memory phenotypes. These seem to be induced by a host-specific food source and persist for several generations. The establishment of induced memory responses might confer an advantage in bacteria that live in fluctuating and challenging environments.

Materials and methods

Strains used in this study

The 414 *P. aeruginosa* isolates (Fig. 1a) that were included in this study were collected at five major sampling sites from clinical microbiology laboratories of hospitals across Germany and at sampling sites in Spain, Hungary, and Romania. In addition, *P. aeruginosa* isolates were collected in German private practice laboratories or provided by strain collection curators [28]. For all of these isolates, transcriptional profiles were recorded in vitro under one standard environmental condition (lysogeny broth (LB)

medium $OD_{600} = 2$) with the aim to correlate gene expression to virulence (this study) as well as to antibiotic resistance phenotypes (to be published elsewhere). Isolate CH2658 was chosen for in vivo and in vitro evolution experiments in this study, because this isolate exhibited a low virulence phenotype but still survived the passages in the larvae. This isolate was obtained from the lab of P. Gastmeier, Charite Berlin, Germany. The in vivo-passaged strains CH2658 I–IV correspond to passages 1–4. The last passage CH2658 IV is referred to as the “evolved strain.” The evolved strain was passaged in LB (4 days, two passages a day) to generate the revertants CH2658 Rev1–4 corresponding to samples from day 1 to 4. The last passage CH2658 Rev4 is referred to as “revertant”. In addition, the clinical isolate was passaged under in vitro conditions in the presence of linolenic acid (Roth) with (CH2658 Lil + P) and without paraffin (CH2658 Lil) in duplicates. As controls, CH2658 was passaged in LB (CH2658 LB) and in LB supplemented with paraffin (CH2658 LB + P). The in vitro passage experiment was conducted for 4 days and two passages a day (in duplicates).

G. mellonella infection and passaging experiments

Based on visual inspection, healthy *G. mellonella* larvae (faunatopics, Germany) were selected for the infection experiments. To prepare the inoculum, bacteria were grown overnight, washed in sterile phosphate-buffered saline (PBS), and adjusted to a final concentration of 5000 cells/ml by serial dilution in PBS. For each individual experiment, ten larvae were infected in parallel by injecting 20 µl of the *P. aeruginosa* inoculum (~100 colony-forming units) into the last left proleg using a 500 µl Hamilton syringe and a 30 G needle. PBS was injected as a control. The larvae were incubated at 37 °C. Death was defined by two major criteria, which were assessed by visual inspection at 24, 40, and 48 h following infection: loss of movement in response to stimulation and melanization of the cuticle.

For in vivo passaging, a multiplicity of infection (MOI) of 1000 cells was used (Fig. 1b). After 24 h of incubation of the infected larvae at 37 °C, the hemolymph was collected via puncturing the larvae. The hemolymph was pooled from ten larvae, diluted ten times in PBS, and plated on cetrimid agar to select for *P. aeruginosa* bacteria. Bacterial colonies were washed off the agar for the next infection round (1000 cell bottleneck). In total, four infection rounds were conducted. Glycerol stocks from every passage were frozen at –80 °C. The evolved strain (CH2658 IV) was used to generate revertants (Fig. 1b). Therefore, single colonies of the evolved strain were passaged in LB medium with shaking (180 r.p.m.) at 37 °C in four independent experiments. An inoculum of $OD_{600} = 0.03$ of the LB culture was transferred every 12 h for a total of 4 days. The clinical

Fig. 1 *G. mellonella* as a model system to study *P. aeruginosa* virulence. **a** % Survival values (mean of biological replicates) of larvae infected with overall 414 clinical *P. aeruginosa* isolates were recorded 48 h post infection (pi) (left). For the most virulent isolates, also survival rates 24 h pi were determined (right). The *P. aeruginosa* reference strains, PA14 and PAO1, are highlighted in red. **b** The less virulent clinical isolate CH2658 was passaged four times in the *G. mellonella* larvae to generate the evolved strain CH2658 IV. The

evolved strain CH2658 IV was passaged (eight times) in rich LB medium for 4 days to generate the revertant CH2658 Rev. **c** The survival rates of *G. mellonella* larvae infected with the clinical isolate (CH2658), the evolved strain (CH2658 IV), and the revertant (CH2658 Rev) were recorded in four biological replicates. PBS was injected as a control ($n = 2$). Asterisks indicate parameters that show statistically significant differences ($p < 0.05$) as determined by the Mann–Whitney U-test

isolate was also passaged in LB medium supplemented with 5% of linolenic acid with and without the addition of paraffin. Paraffin was added in order to generate a micro-aerophilic environment. Using an inoculation of $OD_{600} = 0.05$, the bacterial culture was passaged eight times every 12 h in fresh media. The experiment was performed in duplicates (Fig. 6).

DNA and RNA sequencing

For RNA extraction, bacteria were grown in 10 ml LB to early stationary phase ($OD_{600} = 2$). The RNeasy Mini Kit (Qiagen) and Qias shredder columns (Qiagen) were used according to the manufacturer's instruction. Obtained RNA was quality checked using the RNA Nano Kit on an Agilent Bioanalyzer 2100 (Agilent Technologies). Ribosomal RNA was removed using the Ribo-Zero Bacteria Kit (Illumina) and cDNA libraries were generated with the ScriptSeq v2 Kit (Illumina). The samples were sequenced in single-end

mode on an Illumina HiSeq 2500 device (1×50 bp reads). Mapping was performed using a stampy pipeline [29] with the PA14 genome as a reference. The RNA sequencing (RNA-Seq) data of the clinical isolate, all passages, and revertants used in this study were uploaded to NCBI's Gene Expression Omnibus (GSE122938).

The RNA-Seq data from the 414 clinical isolates were uploaded to NCBI's Gene Expression Omnibus (GSE123544). Differential gene expression and principal component analysis (PCA) were performed using the R package DESeq2 (v.1.18.1) (Fig. 5, Supplementary Fig. S7, and Supplementary Table S1) [30]. Normalized reads per gene (nrpg) were calculated by the default settings of the R package DESeq2. Area proportional Venn diagrams were generated using the online tool BioVenn (Fig. 6) [31].

DNA was extracted from cell pellets derived from overnight cultures using the DNeasy Blood & Tissue Kit (Qiagen) and fragmented with the S2/E210 Focused-ultrasonicator (Covaris) to achieve 300 nt-long fragments. Library

preparation was performed using NEBNext Ultra DNA Library Prep Kit for Illumina (NEB) according to the manufacturer's instructions. The samples were sequenced in paired-end mode (2×250 bp reads) on an Illumina HiSeq 2500 device. Clipped reads were mapped to the PA14 reference genome as described for RNA-Seq mapping. All DNA-seq data were uploaded to SRA database under the reference number PRJNA506546. For single-nucleotide polymorphism (SNP) detection, samtools (v.0.1.19) and parSNP (v.1.2) were used with PA14 genome as a reference. After quality filtering, every identified SNP position was re-checked manually in the integrative genome viewer (IGV) (v.2.3.98).

Correlation of virulence phenotypes and gene expression data of 414 clinical isolates

We calculated the differential gene expression between the most virulent and the less virulent clinical isolates using edgeR (v.3.20.1) (Supplementary Table S2) [32]. Therefore, the transcription profiles that were recorded under rich medium conditions of the virulent isolates (0% survival 24 h post infection = 110 isolates) and less virulent isolates (100% survival 48 h post infection = 31 isolates) were treated as replicates, respectively. We also selected groups of clinical isolates, which expressed each individual gene at high and low levels. Therefore, the expression values (nrpg) for each gene (overall 5946 genes) across all clinical isolates were determined and the group of 50 isolates showing the highest and the group of 50 isolates exhibiting the lowest expression of all individual genes were defined (Fig. 3a, upper panel). Next, we assigned the data of the killing efficiency as determined in the *G. mellonella* assay to the 50 high and the 50 low gene expression clinical isolates across all individual genes (Fig. 3a, lower panel). The Wilcoxon–Mann–Whitney *U*-test was applied to identify significant associations between gene expression values and virulence phenotypes (Fig. 3b).

Determination of the generation times required for phenotype reversion

In four independent experiments, 25 000 cells of CH2658 IV were used to inoculate 10 ml of LB medium. Cultures were grown at 37 °C and every 12 h 25 000 cells were transferred into fresh LB medium. The generation time over 3 days in overall six passages was calculated by subtracting the log₂ cell number at the start point by the log₂ cell number after 12 h. We found a mean duplication time of 45 min (Supplementary Fig. S3). Every 12 h, we also plated an aliquot of the cultures on blood agar plates and monitored the colony morphologies. The evolved strain exhibited a smaller colony size and could be distinguished from the original clinical isolate by visual inspection (Supplementary Fig. S4).

Cytotoxicity in A549 cells

A549 cells were seeded into 24-well plates at a density of 5×10^4 cells/well and incubated in Dulbecco's modified Eagle's medium (DMEM) complete (DMEM high glucose (ThermoFisher) supplemented with 2 mM L-glutamine (ThermoFisher), 1× non-essential amino acids (ThermoFisher), 10% fetal bovine serum (Sigma-Aldrich), 100 U/ml Penicillin–Streptomycin (ThermoFisher)) at 37 °C and 5% CO₂ until 90% confluency. The actual cell number per well was determined before infection. *P. aeruginosa* cells were grown to early stationary phase and washed in DMEM complete without phenol red and antibiotics. Bacterial cells were adjusted to an MOI of 10 before they were added to the PBS-washed A549 cells followed by a brief centrifugation (5 min at 500 × *g*). After 3 and 22 h of incubation, the supernatants were collected, and bacterial cells and cell debris were removed by centrifugation. As a positive control, a cytotox sample was treated with lysis buffer 45 min before the collection of supernatant and cytotoxicity of all samples was determined via lactate dehydrogenase (LDH) assay following the manufacturer's instructions (Pierce™ LDH Cytotoxicity Assay Kit; ThermoFisher) (Supplementary Fig. S2).

Biofilm measurement with crystal violet

The crystal violet assay was performed as described previously [33]. Briefly, overnight-grown, PBS-washed bacteria were inoculated in 100 µl of LB (at an OD₆₀₀ of 0.02) in 96-well microtiter plates (flexible plate, PVC, U bottom, BD Biosciences). The plates were incubated with an air-permeable membrane at 37 °C at a humid atmosphere for 24 h. The wells were washed twice with 200 µl distilled H₂O to remove the media and unattached cells. One hundred and fifty microliters of crystal violet staining solution (0.1% m/v in H₂O) was added and incubated for 30 min at room temperature. Wells were washed twice and dried for 30 min at room temperature. To de-stain the biofilm, 200 µl ethanol (95%) was added and incubated for 30 min. One hundred and twenty-five microliters of the ethanol solution was transferred into a fresh 96-well plate (hart plastic, flat bottom) and absorbance at 550 nm was measured (Figs. 4b and 6c, and Supplementary Fig. S6).

Quantification of cyclic-di-GMP levels

Extraction and quantification of cyclic-di-GMP (c-di-GMP) by the use of high-performance liquid chromatography–coupled tandem mass spectrometry was performed as described previously with some modifications [34, 35]. Briefly, 5 ml of a bacterial suspension were collected after 24 h of cultivation in LB at 37 °C. Before extraction [methanol:acetonitrile:water; 2:2:1], isotope-

labeled $^{13}\text{C}^{15}\text{N}$ -c-di-GMP was added as an internal standard. C-di-GMP extracts were stored overnight at -20°C and c-di-GMP concentrations (pmol c-di-GMP/mg protein) were measured in three independent experiments, each analyzed in duplicates (Figs. 4c and 6d). The protein concentration was determined by the use of the Roti Nanoquant solution for Bradford protein assays according to the manufacturer's instructions (Roth).

Motility

Motility assays were performed as previously described [36]. Swimming assays were performed in BM2 medium with 0.3% agar and swarming assays in BM2 medium with 0.5% agar. Plates were incubated at 30°C for 16 h before optical assessment of swimming and swarming activity from two biological replicates (Fig. 4a).

Fatty acid measurement

To determine the composition of fatty acids in *P. aeruginosa*, the cell pellets (grown to an $\text{OD}_{600}=2$) were saponified [MeOH:NaOH (15%) 1:1, 1 h, 100°C], derivatized [MeOH:HCl 10:2, 10 min, 80°C], and then extracted in the organic phase as described previously [37]. Samples were analyzed by gas chromatography on an Agilent 6890N GC with FID (flame ionization detector). Separation of the fatty acids methyl esters were carried out with a Macherey Nagel Optima 5 column (5% phenyl, 95% dimethylpolysiloxane; 50 m length; 0.32 mm inner diameter; 0.25 μm film thickness). Individual fatty acid methyl esters were identified by their retention time compared with standards and quantified as percentages (%) of the total amount of fatty acids per sample (Fig. 4d).

Results

P. aeruginosa clinical isolates exhibit a broad distribution of virulence phenotypes in the *G. mellonella* infection model

In this study, we used the previously established *G. mellonella* infection model [38] to screen overall 414 clinical *P. aeruginosa* strains for their virulence phenotype. As depicted in Fig. 1a, a broad distribution of virulence phenotypes was observed. There were many virulent isolates that killed 100% of the larvae within 48 h and also a proportion of highly virulent isolates that killed 100% of larvae within 24 h post infection. Furthermore, almost less virulent isolates were found as well as virulent phenotypes at all intermediate levels. Interestingly, the two *P.*

aeruginosa reference strains PA14 and PAO1 exhibited a highly virulent phenotype. Both killed the larvae within 24 h.

Virulence can be increased in a low virulent clinical *P. aeruginosa* isolate upon serial passages in the *G. mellonella* infection model

With the aim to study bacterial adaptation during infection of the *G. mellonella* larvae and to evaluate whether virulence can be induced upon infection, we selected a low virulent *P. aeruginosa* clinical isolate (CH2658) and passaged it overall four times in the *G. mellonella* infection model (Fig. 1b). We observed that virulence increased in the passaged strain (CH2658 IV) and reached the virulence potential of the virulent clinical isolates (Fig. 1c and Supplementary Fig. S1). During the passages, killing of the larvae became faster. This could mean that higher cell numbers are reached in shorter time periods, because the bacteria grow faster. Nevertheless, in accordance with an increased production of virulence factors, the evolved strain (CH2658 IV) also exhibited a significantly increased cytotoxicity in A549 lung epithelial cells as compared with its ancestor (Supplementary Fig. S2A), indicating that the expression of virulence factors could also provide the bacteria with additional nutrients, so that they grow faster.

Transcriptional profile of the evolved *P. aeruginosa* isolate

To obtain more insights into the gained virulence traits, we performed RNA-Seq of the clinical isolate (CH2658) and the evolved strain (CH2658 IV). We found an upregulation of the PseudoCAP categories protein secretion/export apparatus, motility and attachment, and secreted factors (Fig. 2). More specifically, we found higher expression of genes involved in type III secretion (T3SS) in the evolved strain and an enrichment of pili gene expression (a complete list of differentially expressed genes can be found in Supplementary Table S1). Furthermore, genes involved in glycerol metabolism and uptake were upregulated. Overall, less genes (120) were upregulated and more genes (463) were downregulated in the evolved strain ($\log_2$ fold change ≥ 1.5 , p -value < 0.05 , Supplementary Table S1). Among the downregulated genes we found, e.g., genes involved in the production of extracellular matrix (*pslO*, *pslN*, *pslJ*, *algL*, *algG*, *algI*, *alg8*) or glycogen metabolism (*glgA*, *glgB*, *glgP*, *glgX*). In addition, genes involved in general metabolism were downregulated in the CH2658 IV strain, e.g., genes involved in carbon compound catabolism, chemotaxis, adaption, and protection or energy metabolism (Supplementary Table S1 and Fig. 2).

Fig. 2 Transcriptional analysis uncovers phenotypic switching of CH2658 IV. Genes that were differentially expressed under rich medium conditions ($\log_2$ fold change ≥ 1.5) between the evolved CH2658 IV strain and the ancestor clinical isolate CH2658 were assigned to PseudoCAP categories (a complete list of the differentially expressed genes can be found in Supplementary Table S1). The ratio of the abundance of differentially regulated genes (blue, upregulated;

red, downregulated) within the distinct PseudoCAP categories to the abundance of genes in the respective category was determined, as well as the ratio of the abundance of genes within the respective category to the total number of expressed genes. The enrichment factor depicted here represents the ratio of these two values. Asterisks indicate parameters that show statistically significant differences ($p < 0.05$) as determined by hypergeometrical distribution

The evolved *P. aeruginosa* isolate adopts expression of genes that can be linked to a virulence phenotype in the *G. mellonella* model

We next were interested to evaluate the contribution of the transcriptionally reprogrammed genes to the virulence phenotype in the insect larvae. For all 414 clinical strains, which have been tested in this study for their virulence phenotype in the *G. mellonella* model (Fig. 1), we have also generated in vitro transcriptional profiles recorded under rich medium conditions. Here we selected from the group of 414 clinical isolates the 110 most virulent and the 31 least virulent strains (see Material and Methods section for the selection criteria of the clinical isolates) and determined the differentially expressed genes between these two groups. We could show that in accordance with the gene expression profile of the in vivo evolved isolate, the highly virulent clinical isolates upregulate T3SS-related genes, whereas genes important for alginate production are downregulated (alginate operon PA14_18470-18565, *algA*, *algB*, *algD*, *algF*, *algI*, *algJ*; Supplementary Table S2).

Next, we aimed at elucidating which single genes have the highest impact on the bacterial virulence phenotype. Figure 3 depicts the contribution of the expression of 5946 *P. aeruginosa* genes to the virulence phenotype in the *G. mellonella* model. Therefore, clinical isolates that express the individual

P. aeruginosa genes at high vs. low levels were identified and the difference in the virulence potential between the two groups of clinical isolates was assessed. The expression of 389 genes could be positively associated with bacterial virulence in the *G. mellonella* assay, whereas the expression of 534 genes was negatively associated with bacterial virulence (Supplementary Table S4). Figure 3b highlights those genes that were found in this study to be differentially regulated in the evolved strain. Clearly, we found many genes that were shown to be upregulated in the evolved strain, and that were also highly expressed in virulent clinical isolates as compared with the less virulent ones, e.g., the expression patterns of T3SS-related genes and *pil* genes were significantly correlated to the virulence phenotype (Fig. 3c). Vice versa, we also found genes such as the *alg* genes, which were downregulated in the evolved strain as well as in the clinical isolates that exhibited a high virulence phenotype.

Phenotypic change was not linked to genetic variation

In order to identify genomic mutations that might underlie the establishment of the more virulent phenotype within the in vivo evolved strain, we extracted DNA from every in vivo passage (CH2658 I–IV) and sequenced the DNA. The genomes were sequenced at overall 88 × coverage

Fig. 3 Correlation of virulence phenotypes and gene expression data of 414 clinical isolates. **a** The 414 clinical isolates were ranked according to the expression levels of single genes (normalized reads per gene, total of 5946 PA14 genes) as exemplarily shown for three genes (*alg8*, *rplD*, *pilN*) (a, upper row). The 50 isolates that exhibited the highest and lowest expression values of each single gene (indicated in red and blue, respectively) were chosen and their virulence phenotype (survival rate of *G. mellonella* larvae 48 h pi) was assigned (a, lower row). The fold change in the difference of the virulence potential between the groups of high vs. low gene expression was determined (Wilcoxon–Mann–Whitney *U*-test with corrected *p*-values (FDR)). **b** Volcano plot showing the fold difference (y-axis) and the

corresponding *p*-values (x axis) in the virulence potential of the groups of 50 clinical isolates expressing the individual *P. aeruginosa* genes at high vs. low levels (each dot corresponds to one individual *P. aeruginosa* gene). Significant differences in virulence potentials are depicted in blue. Genes that were also found to be differentially regulated (fold change ≥ 1.5) in the *G. mellonella* evolved strain vs. the ancestor isolate CH2658 are highlighted in red (upregulated in the evolved isolate) and black (downregulated in the evolved isolate). **c** The most significant genes that are contributing to virulence in 414 clinical isolates and at the same time were found to be differentially regulated in the *G. mellonella* evolved strain are listed. Downregulated genes and upregulated genes in the evolved strain are shown

(mean value). ParSNP-based SNP calling revealed in total six SNPs between the clinical isolate CH2658 and the evolved strain CH2658 IV. However, manual re-checking in the IGV revealed that all of them were located in regions of low genome coverage and thus were defined as false positive. Thus, genetic variations do not seem to be the reason for the phenotypic change.

Persistence of the induced virulence phenotype despite a change in the conditions that induced the phenotype

To test whether the virulent phenotype of the evolved strain was stable, we cultivated the strain in rich LB medium over a time period of 4 days (Fig. 1b). Clearly as depicted in

Fig. 1c, when the larvae were infected with this revertant isolate (CH2658 Rev), the *G. mellonella* survival rate increased again to the level of the ancestor isolate. The revertant also exhibited the low cytotoxicity phenotype of the original clinical isolate in the A549 lung epithelial cell infection model (Supplementary Fig. S2A). We next monitored the generation time necessary for the evolved strain to revert back to the low virulence level of the ancestor isolate (Supplementary Fig. S3). Therefore, we took advantage of the finding that the evolved strain exhibited a smaller colony morphology on agar plates than compared with the original clinical CH2658 strain or the CH2658 Rev (Supplementary Fig. S4). We calculated the ratios between small and large colony morphologies, and found that after 38 generations ~50% of the evolved strain reverted to the ancestor clinical isolate phenotype. Our results thus suggest a stably induced virulence phenotype that persists in the clinical isolate for several generations despite the change in the conditions that first induced this phenotype. As a control, we selected small and large colonies from the same plate after 1 and 3 days of LB passage and tested them for their *G. mellonella* virulence phenotype (Supplementary

Fig. S5; two small and large colonies per plate, respectively). Whereas the small colonies exhibited a virulent phenotype, large colonies showed a less virulent phenotype similar to the CH2658 isolate and the revertant.

Phenotypic characterization of the evolved *P. aeruginosa* isolate

We evaluated whether the evolved transcriptional phenotype of CH2658 IV was associated with distinct motility phenotypes and/or altered biofilm formation. Swimming and swarming were increased in the evolved strain (Fig. 4a), whereas the strain produced lower biofilm levels as determined by the use of the crystal violet assay (Fig. 4b). Replica passages of CH2658, which were evolved in the *G. mellonella* larvae in independent experiments, exhibited the same phenotypes and showed a low biofilm production that increased again to the levels of the original clinical isolate after serial passages in rich LB conditions (Supplementary Fig. S6), indicating that the CH2658 IV phenotype evolved repeatedly after passaging and was not a rare event that got picked up accidentally. We also analyzed the

Fig. 4 Phenotypic characterization of the evolved strain. **a** Swarming and swimming motility of the clinical isolate (CH2658), the evolved strain (CH2658 IV), and the revertant (CH2658 Rev) was assessed following 16 h of incubation in BM2 medium at 30 °C under humid conditions. One representative example of two biological replicates is shown. **b** Biofilm formation was analyzed using the crystal violet staining assay ($n = 16$). **c** C-di-GMP concentrations were quantified using LC-MS for all *G. mellonella* passages (1–4) as well as for all LB

passages (1–4). Thereby, the *G. mellonella* passage 4 corresponds to the evolved strain (CH2658 IV) and the LB passage 4 to the revertant (CH2658 Rev) ($n = 6$). **d** Fatty acid composition was measured using gas chromatography in combination with a flame ionization detector (FID) ($n = 3$). Asterisks indicate parameters that show statistically significant differences ($p < 0.05$) as determined by the Mann–Whitney *U*-test

intracellular levels of the global second messenger c-di-GMP, which is known to govern virulence, motility, and biofilm phenotypes [39]. As shown in Fig. 4c, the c-di-GMP concentration decreased with every passage in the *G. mellonella* larvae, whereas the c-di-GMP concentration increased again stepwise upon cultivation of the evolved strain in LB medium. We furthermore extracted fatty acids of the bacterial membrane and recorded their composition in the wild type, revertant, and the evolved strain by the use of gas chromatography in combination with a FID. However, we could not observe significant differences in the evolved strain as compared with the clinical isolate and the revertant (Fig. 4d).

To obtain more insights into the evolution of virulence in our clinical isolate, we compared RNA-Seq data of the clinical isolate (CH2658), the evolved strain (CH2658 IV), and the revertant (CH2658 Rev, passage 4). We also included all isolates that were sampled after every single passage in the in vivo (CH2658 I–III) and in vitro conditions (CH2658 Rev1–3). As demonstrated in the PCA analysis (Fig. 5), the transcriptional profiles of the *G. mellonella* larvae passages I and II were quite similar and still close to the ancestor clinical isolate, whereas the profiles of passage III and IV produced distinct clusters. Strikingly, the transcription profiles of the revertants followed the corresponding in vivo passages on each step backwards.

Passage of the clinical isolate in the presence of linolenic acid mimics the *G. mellonella* evolved phenotype

We hypothesized that the particularly high polyunsaturated fatty acid composition in the *G. mellonella* larvae might serve as an environmental cue, which could be involved in the establishment of a memory virulence phenotype. We therefore evaluated whether we could evolve a virulent phenotype in the clinical isolate, if the isolate is passaged in

medium supplemented with polyunsaturated fatty acids. We therefore added 5% of linolenic acid (18:2 ω 6,9) to LB medium. Linolenic acid is a major component of the polyunsaturated fatty acid fraction in the family of lepidoptera (up to 45% of the fatty acids are linolenic acids [27]). We furthermore passaged the clinical isolate in linolenic acid-supplemented LB medium with an additional overlay of paraffin oil in order to mimic the microaerophilic environment during the infection process within the insect host. After passaging the clinical isolate eight times in the presence of linolenic acid with and without paraffin, the gene expression profile was recorded. As depicted in Supplementary Fig. S7, the global transcriptional profile of the in vivo and the in vitro linolenic acid-passaged clinical isolate clearly clustered in the PCA analysis. The genes that were commonly differentially regulated in the in vivo and the in vitro conditions are shown in Fig. 6a (and in Supplementary Table S3). Strikingly, we found an overlap of genes involved in the T3SS and glycerol metabolism. Furthermore, genes involved in biofilm formation (*alg*, *psl*) and in starch and sucrose metabolism or fatty acid metabolism were commonly downregulated in the evolved strains as compared with the clinical isolate independent on whether they evolved in the *G. mellonella* larvae or in linoleic acid-supplemented medium with or without paraffin. Of note, the colony morphology did not change in the in vitro linolenic acid-passaged clinical isolate.

Interestingly, the in vitro (in the presence of linolenic acid)-evolved strains were able to kill the *G. mellonella* larvae to a similar degree as the in vivo-evolved strain (Fig. 6b). They were also more cytotoxic as determined in a cell culture experiment using A549 lung epithelial cells (Supplementary Fig. S2B). Furthermore, the in vitro-passaged isolates produced less biofilm, even less than the in vivo-evolved strain (Fig. 6c). This was in accordance with the finding that the supplementation of linolenic acid over several generations resulted in a lower production of c-

Fig. 5 Transcriptional analysis uncovers phenotypic switching during passages in vivo and in vitro. PCA plot of transcriptional data of all in vivo and in vitro passages grown under rich medium conditions ($n = 2$). The clinical isolate (CH2658) and the in vivo evolved strains from passage 1–4 (CH2658_I–IV) are highlighted in blue and the in vitro passages (1–4) of the revertant (CH2658_Rev1–4) in red (circle and arrow)

Fig. 6 Induction of a memory response upon passing of *P. aeruginosa* in the presence of linolenic acid. **a** Overlapping up- or down-regulated genes of the in vivo, *G. mellonella* passaged CH2658 IV isolate and the in vitro, LB (supplemented with linolenic acid with and without paraffin) passaged clinical isolate CH2658 as compared with the ancestor clinical isolate CH2658 are depicted in a Venn diagram (BioVenn). **b** Kaplan–Meier plot showing the survival rate of *G. mellonella* larvae infected with the clinical isolate, the evolved

strain, the revertant, and the in vitro passages over time. Mean survival rates from two biological replicates are shown. **c** Biofilm formation was analyzed using crystal violet staining ($n = 16$). **d** C-di-GMP concentrations were quantified using LC-MS ($n = 6$). Asterisks indicate parameters that show statistically significant differences ($p < 0.05$) as determined by the Mann–Whitney *U*-test. CH2658 Lil = clinical isolate passaged in LB with 5% linolenic acid, CH2658 LB = clinical isolate passage in LB, P = cultures were overlaid with paraffin

di-GMP, even lower than in the in vivo-evolved strain (Fig. 6d). As a control, we passaged the clinical isolate under normal LB conditions with and without the addition of paraffin. As opposed to the passages that were cultured in the presence of linoleic acid, these passaged isolates did not become more virulent nor did they exhibit decreased biofilm forming capabilities (Fig. 6c, d).

Discussion

The broad ecological success of the opportunistic pathogen *P. aeruginosa* is based on its remarkable capability to adapt to a range of very different growth conditions. One exceedingly challenging environment is the habitat of the eukaryotic host during an infection process. This habitat provides strong selection pressures for adaptive changes. In this study we describe an adaptive mechanism that adds to the well described long-term evolutionary adaptations and short-term individual adaptations. Upon passaging in the

G. mellonella insect host, *P. aeruginosa* adopts a phenotypic response that is characterized by switching to a virulence phenotype. Remarkably, the transcriptionally reprogrammed phenotype was not linked to the acquisition of genetic variations and was sustained for several generations upon cultivation of the ex vivo virulence-adapted *P. aeruginosa* cells under rich medium conditions. The host-induced virulence memory response could be reproduced in bacteria that were passaged in medium supplemented with excess linolenic acid, a long-chain polyunsaturated fatty acid. The *G. mellonella* larvae hemolymph is characterized by a high proportion of triglycerides, diacylglycerols, trehalose, and glycogen, which are released from the fat body [40–42], as well as polyunsaturated fatty acids, the majority of which are linolenic acids [27]. Our results thus indicate that the memory response of *P. aeruginosa* could be induced by exposure to a host-characteristic fatty acid composition. There are several well-described host-specific environmental factors that have an impact on and drive bacterial pathogenicity. These include the higher host

temperature, scarcity of iron, and alterations in the concentration of phosphate, pH, oxygen tension, and osmolality [43–46]. Furthermore, there is increasing evidence that the host-specific fatty acid composition during an infection process can be added to the list of factors that are sensed by the bacteria to induce a virulence phenotype. Host- and in vitro-derived exogenous fatty acids were demonstrated to influence bacterial membrane physiology. This resulted in altered phenotypes such as virulence, motility, antimicrobial peptide susceptibility, or biofilm formation [47–52]. It was proposed that exogenous polyunsaturated fatty acids were directly incorporated into the bacterial membrane and thus to affect membrane permeability. However, it was also argued that bacteria import exogenous fatty acids to generate acyl-CoA by β -oxidation, which can be used for endogenous phospholipid biosynthesis [53].

The memorization of cellular responses to past conditions might be important, because some of them are beneficial for future conditions. Especially in very dynamic environments, carry-over effects generate a history-dependent behavior or bacterial memory that were demonstrated to be of value [54–57]. Thereby, memory responses are general strategies to transmit information between generations and can be achieved in multiple ways. In most of the instances, memory is dependent on the stability of RNAs, proteins, or on the circuit of positive feedback loops in gene-regulatory networks. However, also gene copy number variations might be responsible for carry-over effects, as the fitness effect of a gene duplication may be the result of an increase in protein dosage [58]. It was also claimed that numerous molecular steps involved in birth and decay of macromolecules (e.g., mRNA) can produce gestation and senescence periods or memory between these steps [59]. The clinical isolate of our study developed a hysteretic behavior upon passages in the infected insect host. The induced virulence phenotype was mainly characterized by high expression of genes involved in the T3SS system, but also by enhanced motility and reduced biofilm formation. Virulence, motility, and biofilm formation are well known to be governed by changes in the intracellular levels of the universal intracellular signaling molecule c-di-GMP [39, 60]. One might thus speculate that the in vivo-evolved *P. aeruginosa* isolate expresses a hysteretic c-di-GMP signaling behavior, which might be induced by a carry-over of proteins involved in the modulation of the c-di-GMP levels, and which would be fixed even if the *P. aeruginosa* cells were grown under conditions that would not induce the observed c-di-GMP levels [61]. Future studies will have to uncover whether carry-over effects of c-di-GMP modulating proteins into the next generation result in a high- or low-level c-di-GMP and how this could be linked to the exogenous source of polyunsaturated long-chain fatty acids.

In conclusion, our study highlights the sheer diversity of mechanisms employed by bacteria to adapt expression of

virulence in response to the environmental conditions such as those that prevail in the host niche. Efforts to unravel the fundamental mechanisms that drive bacterial adaptation to challenging habitats will continue to be of importance. Especially in the light of increasing antimicrobial resistance, novel anti-virulence strategies to fight acute and chronic *P. aeruginosa* infections might be further explored and developed into valuable novel therapy options [62]. Our results also imply that memory responses responsible for expression of virulence factors that compromise the host or enable the bacterial pathogen to evade host defense strategies might have been underestimated. It seems that in the future more response memories can be expected to be identified as efficient bacterial adaptation mechanisms to challenging environments. It will be an interesting task to elucidate the molecular mechanism that underlie the carry-over effect to induce such memory responses, which ultimately drive survival of bacterial population in changing and challenging habitats.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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