Supplementary Material

Low spatial structure and selection against secreted virulence factors attenuates pathogenicity in *Pseudomonas aeruginosa*

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Supplementary Methods

Supplementary References

Supplementary Figures S1-S6

Supplementary Table S2

Supplementary Tables S1 and S3 are available as separate files.

Supplementary Methods

Starting the experimental evolution

At the start of the experimental evolution, overnight cultures of PAO1-*gfp* were grown under shaken conditions (190-200 rpm) at 37°C for 18 h, washed with NaCl (0.85%) and adjusted to an OD600 of 1.0. After this point, all steps throughout the experimental evolution were conducted at 25°C. For evolution on agar plates and for each replicate line, 50 µL of cell suspension were spread onto a small RDM-Ch agar plate (diameter 60 mm). Approximately 100 age-synchronized L4 stage *C. elegans* nematodes were then added to each plate in the treatment "agar plate with host", and all plates were incubated for 48 h before the first transfer. For evolution in liquid cultures, the same OD-adjusted bacterial suspensions were diluted 10^{-4} into 5 mL of liquid RDM-Ch in 15 mL culture tubes. Approximately 2500 age-synchronized L4 stage *C. elegans* nematodes were then added to each tube for the treatment "liquid culture with host", and all tubes were incubated for 48 h under "rolling" conditions (160 rpm) in a horizontal position to avoid clumping of the worms. The number of nematodes added at the beginning of the experimental evolution (and at each transfer) was calculated based on the total number of bacteria per plate or culture tube and then kept constant throughout the experiment. We aimed to keep the approximate ratio between bacterial cells and nematodes equal across treatments, and since liquid cultures harbored more bacteria than plates, we adjusted the number of nematodes accordingly.

Transfers conducted during experimental evolution

For all agar plates, bacteria were replica-plated to a fresh RDM-Ch plate, using a custom made replica tool covered in sterilized velvet. In the treatment "agar plate with host", the plates containing the nematodes from the previous round were then rinsed off the plate with sterile NaCl

(0.85%), washed thoroughly to avoid additional transfer of bacteria, and 10% of the nematode suspension was transferred to the new plate. Since *P. aeruginosa* is highly virulent towards *C. elegans*, the transferred worms were carcasses. A fresh batch of ~100 synchronized L4 stage nematodes was then added to the plates. For the "liquid culture without host" treatment, 50 µL of the culture was used to inoculate 4.95 mL of fresh RDM-Ch medium. For the "liquid culture with host" treatment, culture tubes were centrifuged slowly (~200 g, 5 min) to pellet the nematodes, and 50 µL of the supernatant (containing the bacteria) was used to inoculate 4.95 mL of fresh RDM-Ch medium. The pelleted nematodes were then washed thoroughly with sterile NaCl (0.85%), and 10% of the nematode suspension was transferred to the new culture tube. Analogous to the agar treatment, most transferred worms were carcasses due to the high virulence levels of *P. aeruginosa*. A fresh batch of ~2500 synchronized L4 nematodes was then added to the tubes.

Killing Assays in liquid culture

Evolved bacterial populations and the ancestral wildtype strain were re-grown from freezer stocks in LB medium overnight, washed with sterile NaCl (0.85%), adjusted to OD600=1.0 and diluted 10^{-4} into 5 mL of liquid RDM-Ch medium in a 15 mL culture tube. Three replicate tubes were inoculated per tested population. After an incubation period of 48 h (shaken conditions, 160-165 rpm), the OD600 was measured and cells were pelleted through centrifugation. A volume ≤ 500 µL of the supernatant was removed, corresponding to the volume containing ~2500 synchronized L4 nematodes that were subsequently added. Culture tubes were then incubated for 48 h under "rolling" conditions at 160 rpm in a horizontal position to avoid clumping of the worms. At 24 h and 48 h after adding the nematodes, the level of virulence was determined by counting the fraction of dead worms. Small aliquots were taken from the main culture and dropped onto an NGM plate.

After a short drying period, nematodes were prodded repeatedly with a metal rod and counted as dead if they did not show any signs of movement. Dead worms were immediately removed to avoid double counting.

Killing Assays on agar plates

Evolved bacterial populations and the ancestral wildtype strain were re-grown from freezer stock in LB medium overnight, washed with sterile NaCl (0.85%), adjusted to OD600=1.0 and 50 µL were spread on RDM-Ch agar plates. Six replicate plates were inoculated per tested population. Plates were incubated for 48 h, and an aliquot of synchronized L4 nematodes suspended in liquid was then added to the plates. The nematodes had been previously starved on empty NGM plates for 24 h. The starting number of nematodes ranged from 20 to 60 worms per plate and was immediately determined by manual counting. Plates were then incubated further and at 24 h and 48 h after adding the nematodes, the level of virulence was determined by counting the number of dead worms on the plates, as described for the killing assay in liquid culture. For both killing assays, each individual liquid culture and plate was visually checked for egg or L1 larvae development and we never observed any live larvae. We can therefore assume that the nematodes did not successfully reproduce during these experiments.

Freezing of evolved populations

At the end of the experimental evolution, evolved populations were frozen for further analysis as follows. For the two agar plates treatments, the bacterial lawn was washed off with sterile NaCl (0.85%), mixed vigorously, diluted 10^{-3} into 3 mL of liquid LB medium in 6-well plates, and incubated under shaken conditions (100 rpm) for 18 h. For the “liquid culture with host” treatment,

culture tubes were first centrifuged slowly (~200 g, 5 min) to pellet the nematodes. Then, 25 µL of the supernatant (containing bacteria) was used to inoculate 2.475 mL liquid LB medium in 6-well plates. For the "liquid culture without host" treatment, 25 µL of the bacterial culture was used to inoculate 2.475 mL liquid LB medium in 6-well plates. All plates were then incubated under shaken conditions (100 rpm) for 18 h. Finally, 900 µL of each well was mixed 1:1 with sterile glycerol (85%) and frozen at -80°C in separate cryotubes.

Isolation of single clones

To isolate single clones, evolved bacterial populations were re-grown from freezer stock in 3 mL LB medium for 20 h (160 rpm) and adjusted to OD600=1.0. Then, 200 µL of 10^{-6} and 10^{-7} dilutions were spread on large LB agar plates (diameter 150 mm), and plates were incubated at room temperature (~20-25°C) for 48 h. Twenty colonies were then randomly picked for each population and inoculated into 100 µL LB medium in a 96-well plate. Plates were incubated for 24 h under shaken conditions (165 rpm) before adding 100 µL sterile glycerol (85%) to each well, sealing the plates with adhesive foil and freezing at -80°C.

Pyoverdine measurements

Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, cultures were first diluted 10^{-2} in NaCl (0.85%) and then 10^{-2} into liquid RDM-Ch to a final volume of 200 µL in a 96-well plate. Plates were then incubated for 24 h under shaken conditions (165 rpm) and OD600 and pyoverdine-specific fluorescence (emission 400 nm, excitation 460 nm) were measured in a plate reader through single endpoint measurements. Multiple wells inoculated with the ancestral wildtype as well as blank medium

controls were included in every plate. Additionally, the pyoverdine knockout mutant PAO1-Δ*pvdD-gfp* was included as a negative control for pyoverdine fluorescence.

Pyocyanin measurements

Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, cultures were first diluted 10^{-2} in NaCl (0.85%) and then 10^{-2} into liquid LB to a final volume of 1 mL in 24-well plates. Plates were then incubated for 24 h under shaken conditions (165 rpm). The well content was then transferred to 1.5 mL reaction tubes, vortexed thoroughly, and centrifuged to pellet bacterial cells. From each tube, three aliquots of 150 µL of the cell-free supernatant were then transferred to 96-well plates, and pyocyanin was quantified by measuring OD at 691 nm in a plate reader. Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the Rhl-quorum-sensing deficient knockout mutant PAO1-Δ*rhlR* was included as a negative control for pyocyanin production.

Protease measurements

Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, 1 µL of bacterial culture was dropped into a single well of a 24-well plate filled with skim milk agar (5 gL^{-1} LB, 4% (m/V) skim milk powder, 15 gL^{-1} agar) and plates were incubated for 20 h. Pictures of the plates were then taken with a standard digital camera and analyzed with the Image Analysis Software *ImageJ*. The diameter of the clear halo around the bacterial colony and the diameter of the colony itself was measured, and protease production was calculated using the following formula:

$$relative\ protease\ production = \frac{(diameter(halo) - diameter(colony))}{diameter(colony)}.$$

Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the Las-quorum-sensing deficient knockout mutant PAO1-Δ*lasR* was included as a negative control for protease production.

Biofilm measurements

Single clones were re-grown from freezer stocks in 200 µL LB medium for 20 h (165 rpm) in 96-well plates. Then, for each well, the air liquid biofilm was manually removed from the surface with a sterile pipette tip. Cultures were then diluted 10^{-2} into 100 µL LB medium in a 96-well round bottom plate (No. 83.3925.500, Sarstedt, Germany) and incubated under static conditions for 24 h. After removal of the air liquid biofilm, the growth medium containing the planktonic cells was transferred to a fresh flat-bottom 96-well plate and OD was measured at 550 nm in a plate reader. In the plate containing the cells attached to the plastic surface, 100 µL of crystal violet (0.1%) was added to each well and plates were incubated at room temperature for 30 min. Then, the wells were carefully washed several times with ddH_2O, left to dry at room temperature for 30 min, and 120 µL DMSO was added to each well before a final incubation step of 20 min at room temperature. Finally, OD was measured at 570 nm in a plate reader, and the production of surface-attached biofilms was quantified by calculating the "Biofilm Index" (OD570/OD550) for each well [1]. Multiple wells inoculated with the ancestral wildtype as well as blank medium controls were included in every plate. Additionally, the knockout mutant MPAO1-Δ*pelA*-Δ*pslA* was included as a negative control for biofilm production.

Genomic DNA isolation

Clones were re-grown from freezer stocks in 3 mL LB medium in 15 mL culture tubes at 190 rpm for 20-24 h. Genomic DNA was then extracted from 1 mL of culture using the *GenElute*™ *Bacterial Genomic DNA Kit* (Sigma-Aldrich, Switzerland) according to the manufacturer's instructions. At the final step of the isolation protocol, the DNA was eluted in TRIS-HCl without the addition of EDTA to avoid interference with sequencing library preparation. DNA concentration was quantified using the *QuantiFluor® dsDNA System* (Promega, Switzerland) according to the manufacturer's instructions, and diluted to a concentration of 10 ng/µL for use in subsequent library preparation.

Preparation of sequencing library and whole genome sequencing

Sequencing libraries were constructed using the Nextera XT Kit (Illumina, USA). Briefly, 0.8 ng of gDNA per sample was tagmented at 55 °C for 10 min. Libraries were dual-indexed and amplified in the subsequent library PCR. Sequencing libraries were cleaned up using cleanNA SPRI beads (GC biotech, Netherlands) according to the manufacturer's protocol. Next, DNA concentration was quantified using the *QuantiFluor® dsDNA System* (Promega, Germany) and equal amounts of library per sample pooled. Finally, the molarity of the library pool was determined using the *dsDNA High Sensitivity Assay* for the *Bioanalyzer 2100* (Agilent Technologies, Germany). Sequencing was performed 2x150 bp by Microsynth (Balgach, Switzerland) on a NextSeq500 (Illumina, USA).

Variant analysis

Annotated variant calls were only retained if more than 80% of reads contained the alternate base

and if quality scores (Phred-scaled probability of sample reads being homozygous reference) were at least 50 (i.e. P ≤ 10E-5). All variants already occurring in the ancestral wildtype strain were discarded for analysis of the evolved clones. Of the 144 sequenced clones, three had to be discarded before analysis due to low coverage, and one for likely being a mixture of two different genotypes due to contamination. Read alignments covering genes with multiple variants (either in the same or different clones) were manually inspected to remove spurious calls in 19 loci (PA2139-PA2140, PA4875-PA4876, PA3503-PA3504, PA2127-PA2128, PA0604-PA0605, PA0366-PA0367, PA0148-PA0149, PA5024, PA4526-PA4527, PA3969a-PA3970, PA1352-PA1353, PA4280.2-PA4280.3, PA2000-PA2001, PA1234-PA1235, PA4838-PA4839, PA2373, PA2232, PA2296, and PA2492).

Analysis of parallelism and order of mutations

We based our calculation of the relative rates of nonsynonymous to synonymous SNPs (dN/dS) on a 25% chance that a random substitution mutation would be synonymous. In the case of the *P. aeruginosa* genomes we analyzed here, out of a total of 16,779,042 possible SNP mutations within the genes (5,593,014 bp of coding sequences, multiplied by three possible mutations in each position), only 4,237,247 SNP mutations would cause a synonymous change.

To infer the order of mutations, we compared the mutations that were called across strains from the 16 populations, and identified 18 loci (i.e. genes or an intergenic regions) that were mutated in at least two populations. The Supplementary Table S3 lists all mutations in the 18 loci that were mutated in at least two populations, and also lists mutations in *pilE pilG*, *pilM*, *pilN*, *pilO*, *pilU*, *pilW*, and *pilZ* that were only mutated in a single population. To order two given mutations in a given strain, we checked whether other strains from the same population carried only one of these

two mutations, as this would indicate that the other mutation appeared second in the same strain. We observed no mutational patterns inconsistent with this model.

Statistical Analysis

To test for differences in evolved levels of virulence and virulence factor production compared to wildtype levels (Fig. 1BC, Fig.2), we used nonparametric Wilcoxon rank sum tests. We chose this test because it is purely based on the order in which observations from two samples fall (the two samples in our case being the wildtype measurements and the evolved clones/populations) and does not assume that the underlying data are normally distributed. To compare treatment effects on evolved levels of virulence (Fig. 1BC), we used linear mixed models as implemented in the R package lme4 (https://CRAN.R-project.org/package=lme4). In this framework, we used spatial structure (high/low) and host presence (yes/no) as main effects and also included the two-way interaction term. Additionally, mixed linear models allow to account for random effects, which explain a certain level of variance in the data, but are not relevant for the biological question addressed. In our analysis of treatment effects on virulence levels, we implemented the replicated populations per evolved lineage as random effect. To compare treatment effects on evolved levels of virulence factor production (Fig. 2), we used Bayesian statistics as implemented in the R package MCMCglmm [2]. The underlying logic of this method matches the one for mixed linear models described above, but it is based on a non-parametric approach, required when the data is not normally distributed, as was the case for the evolved virulence factor production levels. Specifically, the approach performs generalized linear mixed models based on a Markov chain Monte Carlo (MCMC) algorithm. We analyzed spatial structure (high/low) and host presence (yes/no) as fixed effects as well as the interaction between the two factors. The MCMCglmm

method is based on iterative processes and therefore requires the specification of appropriate prior distributions. We chose priors according to the package guidelines (MCMCglmm package course notes; https://cran.r-project.org/web/packages/MCMCglmm). From the posterior distributions generated by the Bayesian iteration, we report P_{MCMC}, representing the Bayesian equivalent of a P-value. Treatment effects were considered significant when $P_{MCMC} \leq 0.05$. Since Bayesian statistics are based on iterative processes, with outcomes slightly differing across runs, we repeated each analysis five times and report mean P_{MCMC} values in the main text and in Table S1. To test for segregation of evolutionary treatments groups (Fig. 3A), we conducted a PERMANOVA analysis. This is a non-parametric statistical test used to compare groups of objects and test the null hypothesis that spatial parameters like centroids and dispersion are equivalent for all groups [3]. To test for correlation between virulence index and evolved virulence levels (Fig. 3B, S4) and between virulence levels in different killing assays (Fig. S1), we used linear models, which means that we only implemented fixed effects and no random effects. To test for the effect of nonsynonymous SNPs and INDELs on virulence factor production (Fig. 5, S6) we used linear models with "gene of interest mutated (yes/no)" as fixed effect. For a complete list of statistical tests conducted, see Table S1. All analyses were performed in R (http://www.r-project.org).

References

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2. Hadfield JD. MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. J Stat Softw. 2010;33(2):1–22.
3. Anderson MJ. Permutational Multivariate Analysis of Variance (PERMANOVA). In:

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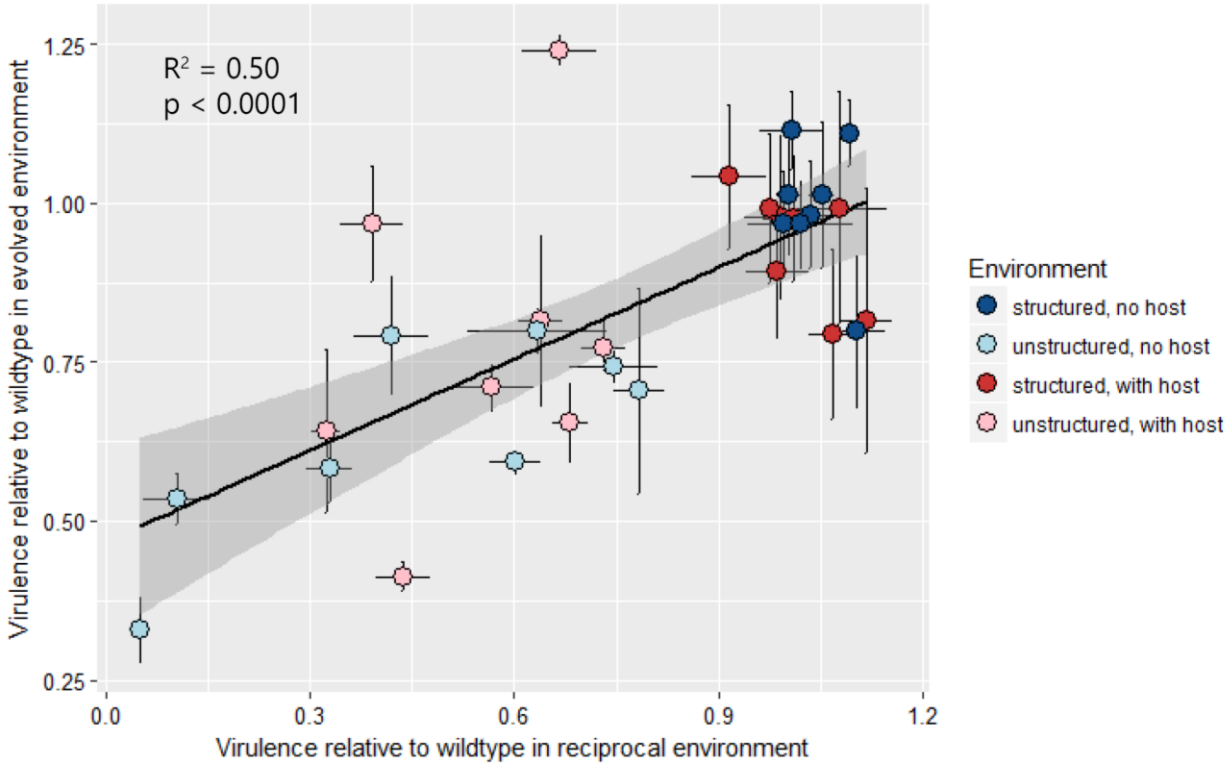


Fig. S1. Virulence measured in two different assays yields highly similar results. 32 evolved populations were tested for their virulence towards the nematode *C. elegans*. *Y* axis shows virulence tested in the environment the populations evolved in: populations that evolved on agar plates tested on agar plates, populations that evolved in liquid culture tested in liquid culture. *X* axis shows virulence when tested in the reciprocal environment: populations that evolved on agar plates tested in liquid culture, populations that evolved in liquid tested on agar plates. Virulence was quantified as percent nematodes killed at 24 h post infection, scaled to the ancestral wildtype, and averaged across three to six replicates per population. Individual dots represent average virulence of a population. Error bars denote the standard error of the mean. See Table S1 for details on statistical analysis.

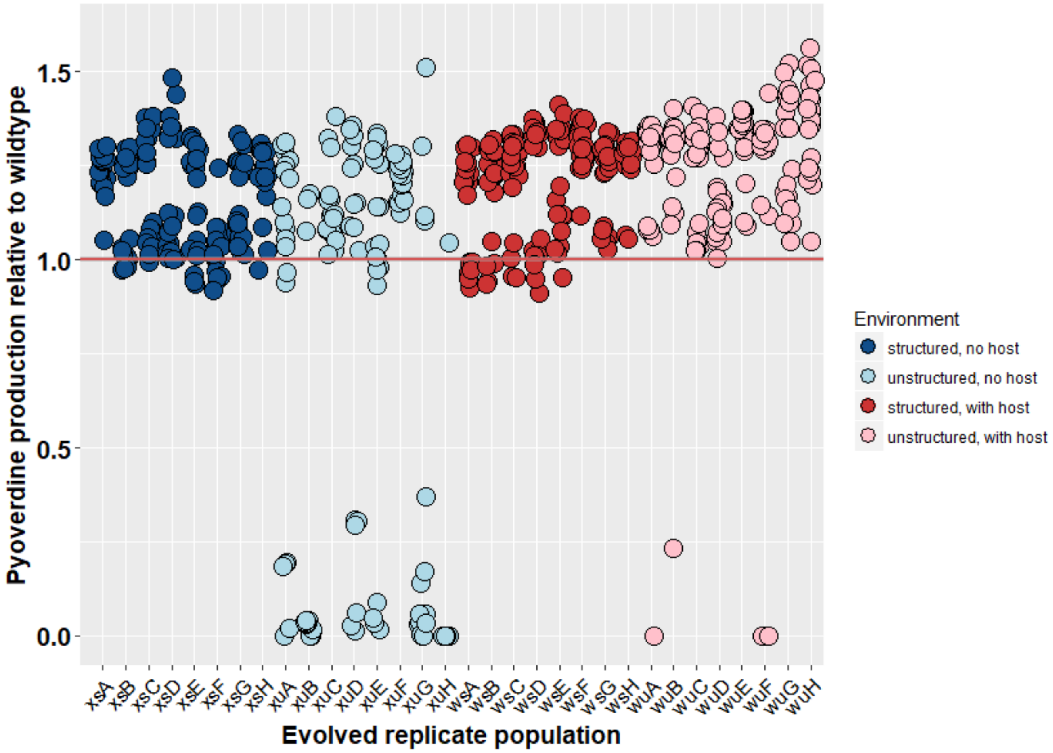


Fig. S2. Loss of pyoverdine production evolved in independent replicate populations. Pyoverdine production levels were determined for 640 evolved *P. aeruginosa* clones (20 clones per evolved population), and compared to the ancestral wildtype (mean ± 95 % confidence intervals indicated as red lines and shaded areas, respectively). Clones with low or no pyoverdine production evolved in 6 out of 8 independent populations in the host-free unstructured environment, and in 2 out of 8 independent populations in the with-host unstructured environment.

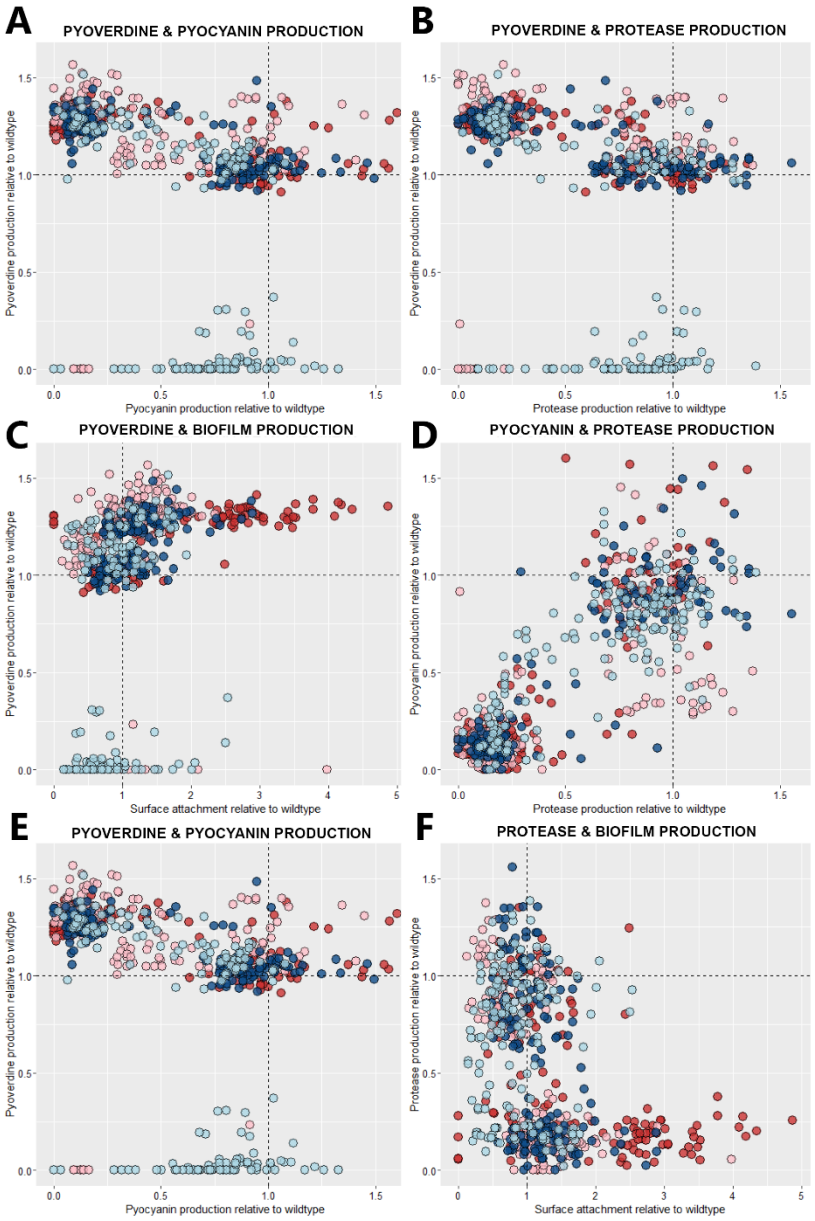


Fig. S3. Pairwise comparisons of the production of different secreted virulence factors. 640 evolved clones were tested for their expression of four secreted virulence factors. Plots each show pairwise comparisons of the production of two virulence factors for all clones. All values scaled to the ancestral wildtype. Colours represent the different environments the populations evolved in. Pyoverdine production against pyocyanin production (A), protease production (B) and biofilm production (C); pyocyanin against protease production (D) and biofilm production (E); protease production against biofilm production (F).

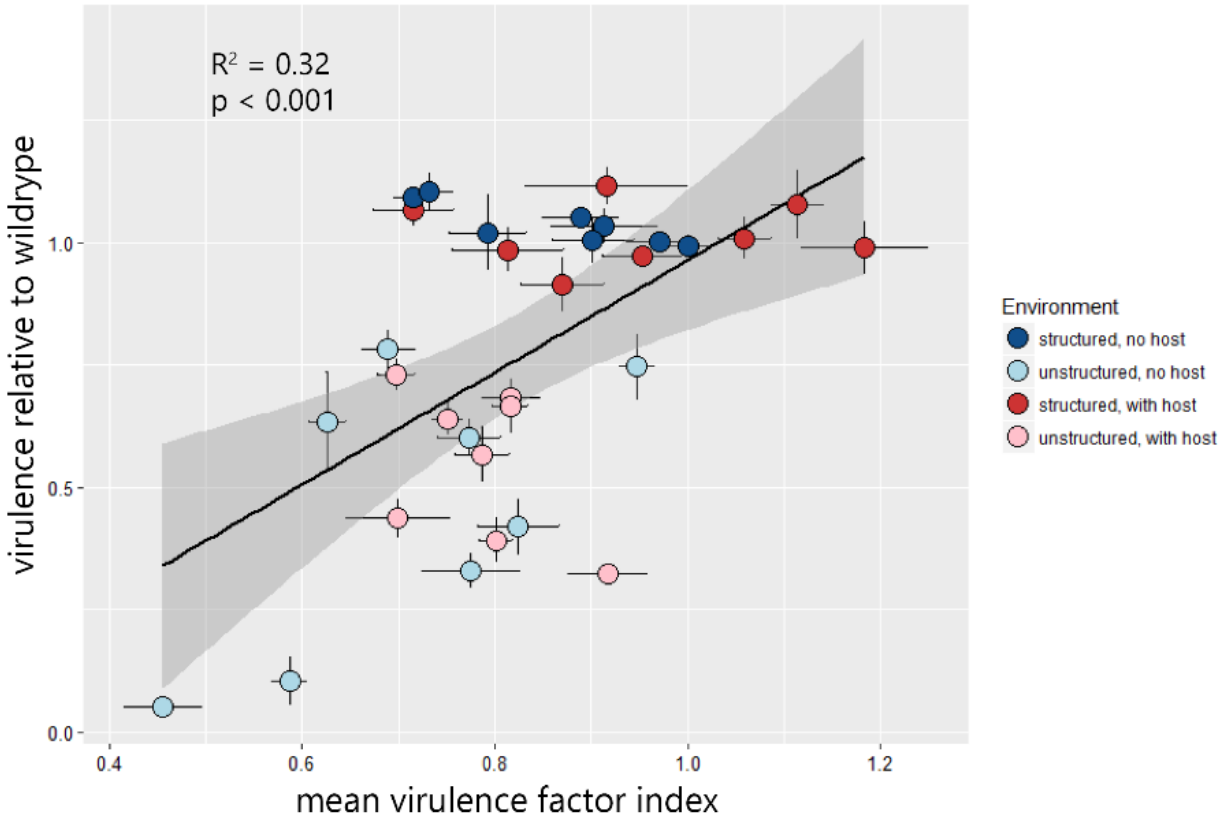
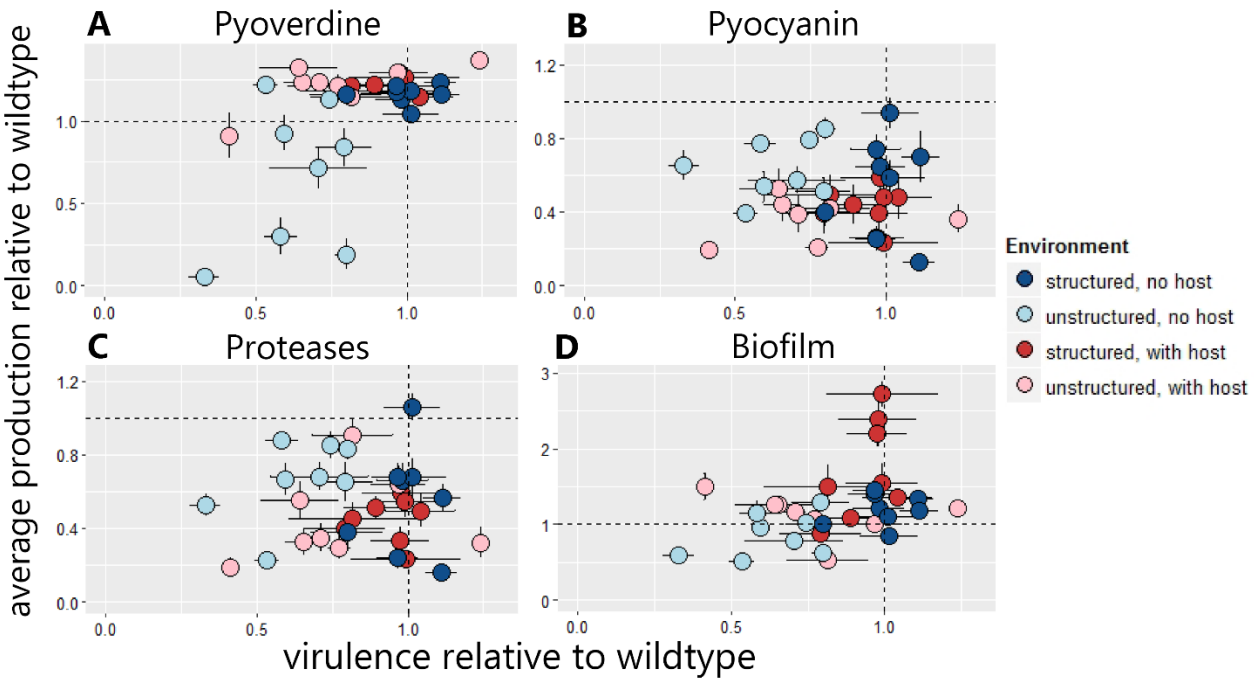


Fig. S4. Decrease in virulence explained by combining the expression profile of four virulence factors in evolved clones. For each of 640 evolved clones, the "virulence factor index" was calculated by summing over production levels of four secreted virulence factors and scaling to the ancestral wildtype. *X* axis shows average virulence indices, while *y* axis shows average virulence levels scaled to the wildtype. Individual dots represent average values across 20 clones for each evolved population, coloured by the environment they evolved in. Virulence was tested in the reciprocal environment: populations that evolved on agar plates tested in liquid culture, populations that evolved in liquid tested on agar plates. Virulence was quantified as percent nematodes killed at 24 h post infection, scaled to the ancestral wildtype, and averaged across three to six replicates per population. Error bars denote the standard error of the mean. See Table S1 for details on statistical analysis.



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285 **Fig. S5. No single virulence factor can fully explain evolved virulence levels.** Virulence levels
286 of 32 evolved populations were determined and plotted against average virulence factor production
287 in 20 evolved clones per population. All values scaled to the ancestral wildtype. Error bars
288 represent the standard error of the mean. (A) Virulence plotted against pyoverdine production. (B)
289 Virulence plotted against pyocyanin production. (C) Virulence plotted against protease production.
290 (D) Virulence plotted against production of surface-attached biofilms. Colours represent the
291 different environments the populations evolved in.

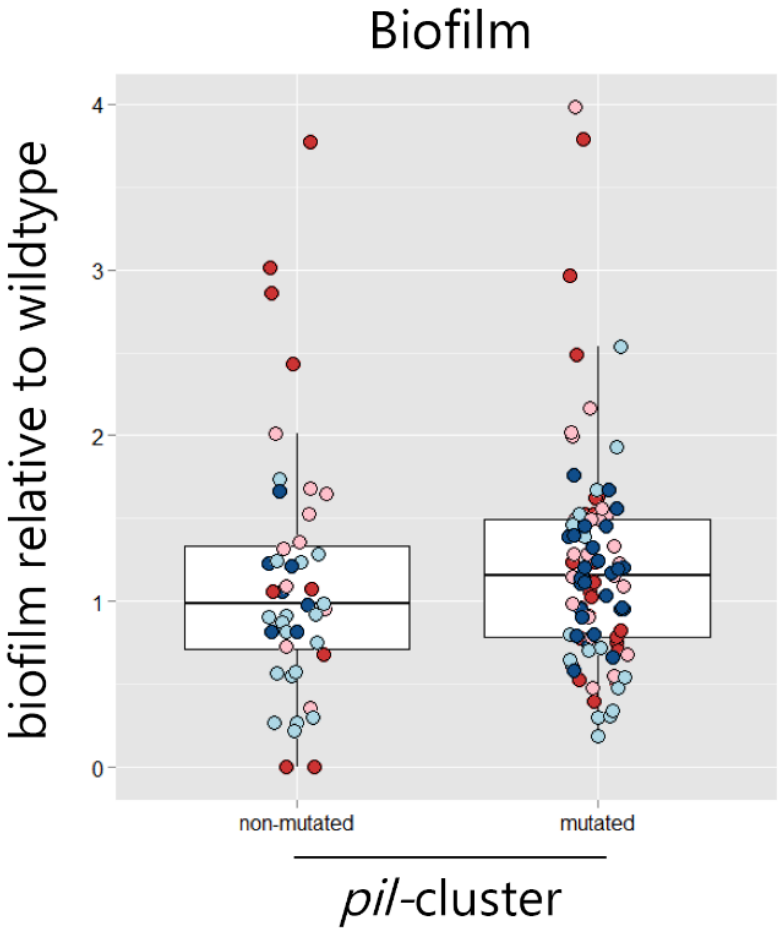

Fig. S6. Biofilm production not affected by mutations in the *pil* gene cluster. Whole genome sequencing was performed on 140 evolved clones, and SNPs and INDELs in genes related to biofilm production were tested for their effect on production levels of surface-attached biofilms. Clones with ≥1 detected SNP or INDEL in the respective gene are labelled "mutated", clones with no SNPs or INDELs detected in this region are labelled "non-mutated". Y axis values are scaled to biofilm production levels of the ancestral wildtype. We did not detect a difference in biofilm production levels in clones with mutations in the *pil* gene cluster (*pilM, pilQ, pilO, pilU, pilD, pilA, pilZ, pilY1, pilW, pilN, pile, pilB, pilS, pilR, pilT, pilG*) when compared to clones without mutations in these genes ($p = 0.64$). See Table S1 for details on statistical analysis.

Table S2. Absolute killing levels in the ancestral wildtype and evolved populations.

	Pre-evolution (ancestral WT)[a]		Post-evolution (evolved populations)[b]			
			no host, structured	no host, unstructured	with host, structured	with host, unstructured
Killing on plates	83.9 %	**Killing in evolved environment[c]**	81.3 %	43.2 %	76.4 %	52.9 %
Killing in liquid	76.2 %	**Killing in reciprocal environment[d]**	87.4 %	40.5%	85.5 %	49.3 %

[a] averaged over 3-6 replicates depending on the killing assay (see methods for details)
[b] averaged over 8 replicate populations per treatment and 3-6 replicates per population (see methods for details)
[c] populations evolved on plates tested on plates, populations evolved in liquid tested in liquid; data shown in Fig. 1B relative to WT
[d] populations evolved on plates tested in liquid, populations evolved in liquid tested on plates; data shown in Fig. 1C relative to WT