Supplementary material

1. Methods for determination of minimal inhibitory concentration (MIC), Minimal biofilm inhibitory concentration (MBIC) and Biofilm preventive concentration (BPC)

The MIC of a stationary-phase planktonic culture was determined by plating different dilutions of the 48h culture on LB plates with different CIP concentrations. The CIP concentration that decreases the size of the population by ≥2 logs was considered the MIC of the stationary-phase population.

The MBIC of CIP for PAO1 colony-biofilms was determined by growing the bacteria for 48 hours on a membrane, as previously described, to form colony- biofilms and transferring the membranes on LB plates with different CIP concentration. The colony biofilms were dispersed after 48 h of incubation and the CFUs/ml of the surviving populations were counted. The CIP concentration that caused a reduction of the biofilm population by≥ 2 logs was considered the MBIC.

The biofilm preventive concentration of CIP for PAO1 colony-biofilm was determined by establishing colony-biofilms on polycarbonate membranes placed on LB plates containing increasing CIP concentrations. The CIP concentration that prevented biofilm formation was considered the BPC.

Table S1. Percentage of population recovered from plates with 0.5 mg/L CIP (A), 1 mg/L CIP (B) and 2 mg/L CIP (C) from CIP-evolved biofilm and planktonic populations during passages (P0 to P6) of the experimental evolution. Mean and SEM of eight lineages are presented.

A.

	CIP_Biofilm (% population recovered from 0.5 mg/L)		**CIP_Planktonic (% population recovered from 0.5 mg/L)**	
	Mean	**SEM**	**Mean**	**SEM**
P0	0.026	0.010	1.077	0.653
P1	18.00	10.31	0.8759	0.4221
P2	17.62	6.982	2.832	0.8542
P3	11.35	3.338	13.00	9.561
P4	13.37	4.444	4.567	3.178
P5	12.77	4.124	3.494	1.515
P6	60.45	21.96	17.85	4.785

B.

	CIP_Biofilm ((% population recovered from 1 mg/L)		**CIP_Planktonic (% population recovered from 1mg/L)**	
	Mean	**SEM**	**Mean**	**SEM**
P0	0.007	0.003	0.089	0.088
P1	2.814	2.572	0.013	0.011
P2	3.706	2.454	0.086	0.086
P3	0.051	0.045	0.012	0.011
P4	0.559	0.365	0.179	0.167
P5	0.578	0.358	0.005	0.003898
P6	1.104	0.429	0.083	0.0828

C.

	CIP_Biofilm (% population recovered from 2 mg/L)		**CIP_Planktonic (% population recovered from 2 mg/L)**	
	Mean	**SEM**	**Mean**	**SEM**
P0	0.0004	± 0.000	0.0000243	± 0.000
P1	0.0300	± 0.020	0.0000148	± 0.000
P2	0.0200	± 0.016	0.0000000	± 0.000
P3	0.0000329	± 0.000	0.0000016	± 0.000
P4	0.0030	± 0.003	0.0000018	± 0.000
P5	0.00008	± 0.000	0.0000002	± 0.000
P6	0.1020	± 0.404	0.0000032	± 0.000

Table S2. Percentage of population recovered from plates with 0.5 mg/L CIP from CTRL-evolved biofilm during passages (P0 to P6) of the experimental evolution. Mean and SEM of eight lineages are presented.

	CTRL_Biofilm (% population recovered from 0.5 mg/L)	
	Mean	**SEM**
P0	37 X10-5	± 18 X10-5
P1	16 X10-4	± 90 X10-5
P2	97 X10-5	± 15 X10-5
P3	15 X10-4	± 55 X10-5
P4	15 X10-4	± 64 X10-5
P5	15 X10-4	± 35 X10-5
P6	85 X10-4	± 41 X10-4

Table S3. The minimum inhibitory concentrations (MICs) of ciprofloxacin (CIP) for selected colonies recovered from each CIP lineage (A. B. C. D) from three evolution experiments (I. II and III) at different passages (P0 to P6). The colonies were selected from 1 and 2 mg/L CIP. The highlighted rows represent the colonies recovered from the lineages used for sequence of the bacterial populations.

Biofilm CIP lineage_ passage no. (Colony no.)_Experiment no._selected concentration	Biofilm_CIP MIC (mg/L)	Planktonic CIP lineage_ passage no. (Colony no.)_Experiment no._selected concentration	Planktonic_CIP MIC (mg/L)
A0 (1)_III_2 mg/L	1,5	A0 (1)_I_2 mg/L	2
A0 (2)_III_2 mg/L	1	A0 (2)_I_2 mg/L	6
A0 (3)_III_2 mg/L	1,5	A0 (3)_I_2 mg/L	6
B0 (1)_III_2 mg/L	2	B0 (1)_I_1 mg/L	1,5
B0 (2)_III_2 mg/L	1,5	B0 (2)_I_1 mg/L	1
B0 (3)_III_2 mg/L	2	B0 (3)_I_1 mg/L	0,5
C0 (1)_III_2 mg/L	0,5	C0 (1)_I_1 mg/L	1
C0 (2)_III_2 mg/L	1	C0 (2)_I_1 mg/L	1
C0 (3)_III_2 mg/L	1	C0 (3)_I_1 mg/L	1
D0 (1)_III_2 mg/L	1,5	D0 (1)_I_1 mg/L	8
D0 (2)_III_2 mg/L	1	D0 (2)_I_1 mg/L	1,5
D0 (3)_III_2 mg/L	1,5	D0 (3)_I_1 mg/L	32
A0 (1)_I_1 mg/L	4	A0 (1)_II_1 mg/L	3
A0 (2)_I_1 mg/L	3	A0 (2)_II_1 mg/L	1
A0 (1)_II_1 mg/L	1	A0 (3)_II_1 mg/L	3
B0 (1)_II_1 mg/L	1,5	C0 (1)_II_1 mg/L	2
B0 (2)_II_1 mg/L	1,5	C0 (2)_II_1 mg/L	3
B0 (3)_II_1 mg/L	1,5	C0 (3)_II_1 mg/L	1,5
D0 (1)_II_1 mg/L	1,5	D0 (1)_II_1 mg/L	2
D0 (2)_II_1 mg/L	1,5	D0 (2)_II_1 mg/L	32
D0 (3)_II_1 mg/L	2	D0 (3)_II_1 mg/L	8
B1 (1)_III_1 mg/L	1,5	A1 (1)_I_2 mg/L	4
B1 (2)_III_1 mg/L	0,75	A1 (2)_I_2 mg/L	4
B1 (3)_III_1 mg/L	1,5	B1 (1)_I_2 mg/L	1,5
C1 (1)_III_1 mg/L	1	B1 (2)_I_2 mg/L	1,5
C1 (2)_III_1 mg/L	1,5	D1 (1)_I_2 mg/L	0,38
C1 (3)_III_1 mg/L	1	D1 (2)_I_2 mg/L	0,125
B2 (1)_III_1 mg/L	2	A2 (1)_I_2 mg/L	2
C2 (1)_III_1 mg/L	1,5	A2 (2)_I_2 mg/L	1
C2 (2)_III_1 mg/L	1	A2 (3)_I_2 mg/L	1,5
C2 (3)_III_1 mg/L	1,5	B2 (1)_I_2 mg/L	4
D2 (1)_III_1 mg/L	2	B2 (2)_I_2 mg/L	3
D2 (2)_III_1 mg/L	1	C2 (1)_I_2 mg/L	0,5
……	……	D2 (1)_I_2 mg/L	3
……	……	D2 (2)_I_2 mg/L	3
A3 (3)_III_1 mg/L	0,75	A3 (1)_I_2 mg/L	0,5
B3 (1)_III_1 mg/L	1	A3 (2)_I_2 mg/L	3
B3 (2)_III_1 mg/L	2	B3 (1)_I_2 mg/L	32
B3 (3)_III_1 mg/L	1,5	B3 (2)_I_2 mg/L	32
C3 (1)_III_1 mg/L	0,75	C3 (1)_I_2 mg/L	12
C3 (2)_III_1 mg/L	1	C3 (2)_I_2 mg/L	8
C3 (3)_III_1 mg/L	1	D3 (1)_I_2 mg/L	0,25
A4 (1)_III_1 mg/L	2	A4 (1)_I_2 mg/L	2
B4 (1)_III_1 mg/L	1,5	A4 (2)_I_2 mg/L	2
B4 (2)_III_1 mg/L	1	B4 (1)_I_2 mg/L	2
B4 (3)_III_1 mg/L	1	B4 (2)_I_2 mg/L	3
D4 (1)_III_1 mg/L	2	C4 (1)_I_2 mg/L	4
D4 (2)_III_1 mg/L	0,75	C4 (2)_I_2 mg/L	6
D4 (3)_III_1 mg/L	2	D4 (1)_I_2 mg/L	0,5
……	……	D4 (2)_I_2 mg/L	0,5
A5 (1)_III_1 mg/L	1	A5 (1)_I_2 mg/L	4
B5 (1)_III_1 mg/L	1	A5 (2)_I_2 mg/L	2
B5 (2)_III_1 mg/L	1,5	B5 (1)_I_2 mg/L	0,75
B5 (3)_III_1 mg/L	0,75	B5 (2)_I_2 mg/L	0,75
C5 (1)_III_1 mg/L	6	B5 (3)_I_2 mg/L	3
D5 (1)_III_1 mg/L	4	C5 (1)_I_2 mg/L	0,75
D5 (2)_III_1 mg/L	1,5	C5 (2)_I_2 mg/L	2
D5 (3)_III_1 mg/L	3	D5 (1)_I_2 mg/L	6
……	……	D5 (2)_I_2 mg/L	6
A6 (1)_III_2 mg/L	2	A6 (1)_I_1 mg/L	0,75
A6 (2)_III_2 mg/L	4	A6 (2)_I_1 mg/L	1
A6 (3)_III_2 mg/L	2	A6 (3)_I_1 mg/L	0,094
B6 (1)_III_2 mg/L	1,5	B6 (1)_I_2 mg/L	32
B6 (2)_III_2 mg/L	6	B6 (2)_I_2 mg/L	32
B6 (3)_III_2 mg/L	1,5	B6 (1)_I_1 mg/L	6
C6 (1)_III_2 mg/L	2	C6 (1)_I_2 mg/L	8
C6 (2)_III_2 mg/L	3	C6 (2)_I_2 mg/L	4
C6 (1)_III_1 mg/L	1,5	C6 (3)_I_2 mg/L	12
D6 (1)_III_2 mg/L	3	D6 (1)_I_1 mg/L	1
D6 (2)_III_2 mg/L	1	D6 (2)_I_1 mg/L	1
D6 (3)_III_2 mg/L	1	D6 (3)_I_1 mg/L	1,5
A6 (3)_I_2 mg/L	3	A6 (1)_II_2 mg/L	8
A6 (4)_I_2 mg/L	4	A6 (2)_II_2 mg/L	8
A6 (5)_I_2 mg/L	1,5	A6 (3)_II_2 mg/L	16
A6 (1)_II_2 mg/L	4	B6 (1)_II_2 mg/L	2
A6 (2)_II_2 mg/L	1,5	B6 (2)_II_2 mg/L	2
A6 (3)_II_2 mg/L	3	B6 (3)_II_2 mg/L	1,5
B6 (1)_II_2 mg/L	6	C6 (1)_II_2 mg/L	32
B6 (2)_II_1 mg/L	2	C6 (2)_II_2 mg/L	3
B6 (3)_II_1 mg/L	6	C6 (3)_II_2 mg/L	32
D6 (1)_II_2 mg/L	4	D6 (1)_II_2 mg/L	4
D6 (2)_II_2 mg/L	1,5	D6 (2)_II_2 mg/L	2
D6 (3)_II_2 mg/L	1	D6 (3)_II_2 mg/L	3

Table S4: Mean coverage of the genome sequencing and the number of non-synonymous mutations in each sequenced evolved population. CTRL control (evolved in the absence of antibiotic). CIP evolved in the presence of 0.1 mg/L CIP.

CTRL Populations	**Coverage**	**Non-synonymous mutations (number)**	**CIP populations**	**Coverage**	**Non-synonymous mutations (number)**
CTRL_Biofilm A	84.15	9	CIP_ Biofilm A	107.16	5
CTRL_Biofilm B	92.83	3	CIP_Biofilm B	91.42	11
CTRL_Biofilm C	90.06	7	CIP_Biofilm C	77.63	20
CTRL_Biofilm D	122.52	2	CIP_Biofilm D	84.71	17
CTRL_Planktonic A	72.17	9	CIP_Planktonic A	105.68	8
CTRL_Planktonic B	91.88	5	CIP_Planktonic B	91.04	9
CTRL_Planktonic C	122.14	7	CIP_Planktonic C	113.22	5
CTRL_Planktonic D	89.98	9	CIP_Planktonic D	151.03	15

Table S5. The list of SNPs in the evolved bacterial populations from control (CTRL) and CIP-evolved (CIP) planktonic and biofilm cultures. The gene name, function, the lineage, the frequency as well as the nucleotide and amino acid changes are presented. Fs frameshift

Supplementary file 2.

TableS6. Twitching motility (mm) of 40 colonies collected from the four lineages (A. B. C. D) of the evolved biofilm and planktonic populations in the presence of sub-inhibitory concentrations of CIP (CIP) or in the control (CTRL) evolved populations. Twitching motility was investigated according to previously described method (1).

Lineage	Colony number	1	2	3	4	5	6	7	8	9	10
CTRL A_Biofilm		36	41	42	43	38	42	42	36	43	45
CTRL B_Biofilm		56	54	58	56	56	58	55	51	59	51
CTRL C_Biofilm		39	35	40	38	41	39	39	44	41	42
CTRL D_Biofilm		53	53	57	59	54	60	48	50	59	60
CTRL A_Planktonic		37	38	32	33	32	44	47	45	43	51
CTRL B_Planktonic		34	42	39	33	39	38	37	42	35	39
CTRL C_Planktonic		44	53	45	55	55	51	44	49	53	55
CTRL D_Planktonic		53	47	56	47	46	54	45	50	46	54
CIP A_Biofilm		0	0	0	0	0	0	0	0	0	0
CIP B_Biofilm		0	0	0	0	0	0	0	0	0	0
CIP C_Biofilm		15	26	24	ND	18	22	22	25	27	25
CIP D_Biofilm		0	0	0	0	0	0	0	0	0	0
CIP A_Planktonic		48	42	48	0	42	36	48	25	32	29
CIP B_Planktonic		0	0	0	0	0	0	0	0	0	0
CIP C_Planktonic		0	0	0	52	0	0	35	0	0	0
CIP D_Planktonic		0	0	0	0	0	0	0	0	0	0

Table S7. Protease activity measured as the diameter (mm) of the clearing zone on skimmed-milk plates surrounding the inoculation spot of 10 colonies, assessed at 48h as described in (2).

Lineage	Colony number	1	2	3	4	5	6	7	8	9	10
PAO1 initial inoculum		10	10	10	-	-	-	-	-	-	-
CTRL A_Biofilm		0	7	10	7	20	10	10	10	24	12
CTRL B_Biofilm		0	0	20	0	0	0	0	0	0	0
CTRL C_Biofilm		0	0	0	0	0	0	0	0	0	0
CTRL D_Biofilm		9	9	9	11	10	10	9	9	10	10

Reference List

1. **Lee B, Haagensen JA, Ciofu O, Andersen JB, Hoiby N, and Molin S.** 2005. Heterogeneity of biofilms formed by nonmucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. *J Clin Microbiol*.**43**: 5247-5255.

2. **Wassermann T, Meinike JK, Ivanyshyn K, Bjarnsholt T, Khademi SM, Jelsbak L, Hoiby N, and Ciofu O.** 2016. The phenotypic evolution of Pseudomonas aeruginosa populations changes in the presence of subinhibitory concentrations of ciprofloxacin. *Microbiology*.**162**: 865-875.