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Supplementary Materials for

CFTR-PTEN–dependent mitochondrial metabolic dysfunction promotes *Pseudomonas aeruginosa* airway infection

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The PDF file includes:

Table S2. Pathoadaptive mutations conserved in all 17 CF *P. aeruginosa*.
Table S3. Accession numbers for *P. aeruginosa* isolates.
Table S4. PAMPs found mutated in all CF *P. aeruginosa* isolates.
Table S5. Sequences of the primers used for qRT-PCR.

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/11/499/eaav4634/DC1)

Data file S1 (Microsoft Excel format). Raw data from figures.

SUPPLEMENTARY MATERIALS

Materials and Methods

Reagents used in this study - For flow cytometry, antibody used were: anti-mouse CD45 AF700 (BioLegend), anti-mouse CD11c Bv605 (BioLegend), anti-mouse SiglecF (BD Bioscience), anti-mouse CD11b AF594 (BioLegend), anti-mouse MHC II APC/Cy7 (BioLegend), anti-mouse Ly6C Bv421 (BioLegend), anti-mouse Ly6G PerCP/Cy5.5 (BioLegend), anti-mouse CD193 FITC (BioLegend), anti-mouse pro-IL-1b PE/Cy7 (eBisocience), anti-mouse PTEN (Abcam), anti-mouse/human IRG1 (Abcam), anti-human SDHA (Abcam), anti-human IDH (Abcam). For Western blots, antibodies used were: anti-mouse actin (Sigma), anti-mouse IL-1β (R&D), anti-mouse HIF1α (Cayman Chemicals), HRP-coupled goat anti-rabbit (Santa Cruz Biotechnology), HRP-coupled goat anti-mouse (Santa Cruz Biotechnology). For Confocal Microscopy, antibodies PTEN (Novus Biologicals), mouse anti-human CFTR (Abcam), rabbit anti-human TOMM20 (Abcam), donkey anti-goat AF488 H+L, donkey anti-mouse AF647 H+L, donkey anti-rabbit AF546 H+L. Succinate was obtained from Sigma. Succinate measurements were performed by using the Abcam Succinate Kit.

***Pseudomonas aeruginosa* strains -** The PAO1 laboratory strain was grown in LB overnight and sub-cultured until exponential phase for infection experiments. 17 host-adapted *P. aeruginosa* strains were recovered between 2012 and 2015 from sputum of a chronic-infected CF patient (p.Q493VfsX10/3849+10kbC>T *CFTR*). These strains were plated in LB agar and their phenotype was characterized regarding SCV and mucoid morphology. These 17 strains were previously characterized (*44*) for growth curves, capacity to form biofilm, pigment production, swimming and swarming motility, and expression of virulence genes. These bacteria were grown in LB and sub-cultured as was PAO1.

ROS production and quantification in bacteria - PAO1 was grown overnight in succinate-free LB media under agitation at 37°C in a sterile tube. OD_{600nm} was measured and an aliquot was spun down and resuspend in 3ml of LB alone, LB + 50mM succinate or LB + 500mM succinate with a final OD_{600nm} of 1.0. Bacteria were agitated in tubes at 37°C for 2h, and an aliquot was washed with cool PBS and resuspended in 5uM of MytoSox red-PBS (Life Technologies) and incubated at 37°C for 20min. After staining, bacteria were washed twice in cold PBS and analyzed by flow cytometry. Non-stained controls were used for each treatment.

CF artificial sputum media - Airway artificial media mimicking CF sputum conditions was prepared as previously described (33). One litter of this media contained 4 g fish DNA, 5 g type-II mucin from porcine stomach, 0.25 g of each of 20 aminoacids, 5.9 mg diethylenetriaminepentaacetic acid (DTPA), 5 g NaCl, 2.2g KCl, 5ml egg yolk emulsion, and pH 6.9 buffered with 1 M Tris (pH 8.5). CF ASM was sterilized with by vacuum-filtration during 2 days in a sterile and closed hood. CF ASM media was stored at 4°C.

Carbon source assimilation by different strains of *P. aeruginosa* - Either PAO1, PAO1*, $PAO1^{Succ}$ (generated in either LB or CF ASM), mucoid 605 or SCV 686 were grown until exponential phase, OD_{600nm} measured and applied to a 96-well plate array where each well contained a single carbon source (PM1 plates, Biolog Inc). For preparation of bacteria, solutions and plates the instructions of the manufacturer were followed. Plates were incubated at 37°C for 48h-72h.

DNA extraction and whole genome sequencing - All isolates were grown overnight in LB broth at 37º C. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer instructions. Genomic DNA libraries were prepped using the Nextera XT kit and sequenced on a HiSeq 2500 sequencer (Illumina) with 125-bp paired-end reads. Genome assembly was done using the paired-end implementation of ABySS.

Single-Nucleotide Polymorphism (SNP) Calling - Comparison of SNPs between isolates was done by means of short-read alignment to the genome of PAO1 as reference (GenBank: AE004091), using the Burrows–Wheeler alignment (bwa) tool (available at: http://bio-bwa.sourceforge.net). SNP calls were made using samtools (available at: http://samtools. sourceforge.net). SNPs that were not listed as heterozygous and had a per base Q score ≥20 were defined as high quality.

Heatmap - Two heatmaps (protein change and no protein change) based on the number of observed SNPs in each genome were built. The SNPs (in one taxon or more) with quality scores <20 and/or listed as “heterozygote” were excluded. In addition, the genomic regions that are identical across the 17 taxa were excluded. For clarity, 29 genomic (nonsynonymous=4; synonymous=25) regions with >3 SNPs were excluded. Genomic regions were clustered using the complete hierarchical clustering method of the pheatmap R package based on the number of

observed SNPs in each genome. The Euclidean distance matrix was used in clustering data for dendrogram generation.

Protein extraction and western blots - Total BAL and lung tissue were removed from WT infected mice after 24h infection. Total cell from either BAL and lungs were washed and resuspended for 30min in 300uL of RIPA buffer containing protease/phosphatase inhibitors at 4°C. Samples were centrifuged for 20min at 15000g and the supernatant was recovered. Samples were prepared, denatured at 37C for 20min and ran in 4-12% gels in MES buffer for actin and IL-1β and MOPS for HIF1α. Membranes were transferred using i-blot and stained overnight with primary antibodies. After that, samples were washed and incubated for 1h with the HRP-tagged secondary antibody. Membranes were revealed and analyzed for protein expression.

Flow cytometry, cell recruitment and intracellular IL-1β staining - BAL and lung tissues were obtained, depleted of red blood cells and the cells recovered. In 96-well plates, cells were stained for viability (DAPI) and for surface cellular markers, such as alveolar macrophages (CD45+CD11b$^{low/-}$SiglecF^{high}CD11c^{+}CD193^{-}Ly6G^{-}Ly6C^{-}), eosinophils (CD45+CD11b$^{low/-}$SiglecF$^{low/-}$CD11c^{-}CD193^{+}Ly6G^{-}Ly6C^{-}), neutrophils (CD45+CD11b^{high}SiglecF$^{low/-}$CD11c^{-}MHCII^{-}CD193^{-}Ly6G^{+}Ly6C$^{low/-}$) and monocytes (CD45+CD11b^{high}SiglecF$^{low/-}$CD11c^{-}MHCII^{-}CD193^{-}Ly6G^{-}Ly6C$^{low/high}$). When indicated, cells were fixed and permeabilized with Fix/Perm kit (eBioscience) and stained intracellularly for pro-IL-1β. Samples were fixed and analyzed in a LSRII machine. Data was analyzed with FlowJo version X.

RNA extraction and qRT-PCR for metabolic and structural genes in *P. aeruginosa* - Strains were grown in succinate-free LB in presence and absence of succinate (80 mg/mL; 500mM) until exponential phase. Total RNA was extracted using RNeasy Mini Kit (QIAGEN) and was treated with DNase (DNA-free, Ambion). The absence of contaminating DNA was checked by conventional PCRs. The RNA concentration was measured using a NanoDrop ND-1000 V3.7.1. cDNA was synthesized using Precision nanoScript Reverse Transcription kit (PrimerDesign) and qPCR was performed with an ABI 7300 Real-Time PCR System (Applied Biosystems) using Power SYBR green PCR master mix (Applied Biosystems). Sequences of the primers used for qRT-PCRs are listed in **table S5**. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method. The *rpsL* gene was used as reference housekeeping gene and *P. aeruginosa* PAO1 strain was used as calibrator.

Metabolomics for nutrients found in BAL of control and infected mice - WT mice were intranasally infected with 10^7 CFU of either PAO1 or an equal mixture of all 17 CF isolates ($5.9x10^5$ CFU each). Control mice were treated with PBS. 24h later, mice were sacrificed and BAL were collected with 3ml of sterile PBS. Samples were immediately placed on ice. Then, samples were diluted with 50% methanol in a 1:1 proportion, mixed and stored at -80C for future metabolomics analysis. Just prior to mass spectrometry, samples were thawed and dried under a stream of N_2 and were resuspended in HPLC-grade water at a 4:1 dilution (relative to the original BAL volume). High-resolution mass spectrometry data were acquired on a Thermo Fisher Exactive Mass spectrometer in negative mode using 25 min reverse phase gradients and ion-pairing chromatography. Metabolites were identified using the known chromatographic retention times of standards, and metabolite signals were quantified using MAVEN. Metabolite signal intensities were used to quantify difference between treatments in BAL.

Antimicrobial susceptibility for host-adapted *P. aeruginosa* - Susceptibility testing was carried out by disc-diffusion agar method. Antipseudomonal agents tested were ticarcillin, piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, doripenem, gentamicin, tobramycin, amikacin, netilmicin, ciprofloxacin, and colistin.

Seahorse experiments - Human monocytes (THP-1 cells) were infected for 3h at multiplicity of infection (MOI)=10 with either PAO1, host adapted SCV 686 or mucoid 605. During the infection cells were analyzed in a Seahorse machine using commercially obtained plates as recommended by the manufacturer to evaluate glycolysis and the oxygen consumption rate.

Succinate measurements in BAL – As indicated, either anesthetized WT C57bl/6, *Ptenl*$^{-/-}$ or *Cftr*$^{\Delta F508/\Delta F508}$-gut corrected mice were infected intranasally with either 1 x 10^7 CFU of laboratory strain PAO1, a total 1 x 10^7 CFU mix of all 17 isolates (equal parts – 5.9 x 10^5 for each), 1 x 10^7 CFU of SCV variant 686, 1 x 10^7 CFU of mucoid strain 605 or a total 1 x 10^7 CFU mix of both 686 + 605 (5 x 10^6 of each). 24h/48h after, BAL was recovered and analyzed for succinate content by using an enzymatic- colorimetric assay (Abcam). Plates were read in a TECAN plate reader. Before the lavage, a 25G needle was introduced through the trachea and the total local respiratory fluid during infection was quantified in some mice. As a representative group, the average volume obtained from a least 10 mice was 15uL (minimal of 1uL and maximum of 50uL). Thus, after 3ml of PBS lavage, the concentration of

succinate quantified in BAL was amplified by a factor of 200 (3ml/15uL), to reflect the average initial concentration. After the 3ml PBS lavage, BAL was spun down at 1400rpm for 10min at 4ºC and supernatants were used for analyses.

Succinate secretion as a function of media - Both PAO1, SCV 686 and mucoid 605 were grown overnight in LB. Then, cells were washed, OD adjusted to 1.0 and a small alliquaot (5uL) subcultured overnight in minimal media M9 supplemented either with α-D-glucose (16mM) or acetate (34mM). Media was also supplemented with $MgS0_4$ and $CaCl_2$ as indicated by the provider. Culture was made in a U-bottom 96-well plate under agitation. Next day, samples were passed to a 96-well flat-bottom plate and read in a TECAN machine OD_{600nm}. In addition, succinate content in the supernatant was quantified by using an enzymatic- colorimetric assay (Abcam).

Quantification of succinate in PBMCs supernatants and human BAL - PBMCs derived from blood of CF patients or healthy volunteers were separated in a ficoll gradient. Cells were washed several times and counted. 400,000 cells were plated in round-bottom 96-well plates and treated either with PBS or WT *P. aeruginosa* for 2h, MOI=10 and in RPMI media without antibiotics. Then, cells were washed and fresh RPMI supplemented with gentamicin was added. Cells were incubated overnight. Next day, supernatants were recovered and stored at -80°C. Succinate in supernatant was measured with a colorimetric assay.

Human BAL from healthy controls and CF patients were obtained from clinics, centrifuged at maximum speed and 5ouL of supernatants were analyzed for succinate accumulation by using the Abcam colorimetric assay.

PTEN KO cell line infection and succinate secretion experiments - Two cells lines lacking PTEN (*PTEN* $^{+/+}$ and *PTEN* $^{-/-}$ HCT116 and DLD1 cells) were plated in 6-well plates, counted, and infected or not at MOI=10 with WT *P. aeruginosa* (PAO1) in antibiotic free media at 37ºC with 5% CO_2. After 2h, cells were washed and incubated in gentamicin-containing media overnight. The next day, 50uL of the supernatant was collected and analyzed for succinate content. Where indicated, cells were fixed, permeabilized and stained intracellularly for IRG1, SDHA and IDH. Cells were analyzed by flow cytometry. In these cells, succinate dehydrogenase activity was analyzed in total protein extracts (Abcam).

CFBE cells and metabolic assays - ΔF508/ΔF508 *CFTR* epithelial cells were transduced with a WT CFTR-coding lentivirus or an empty-control. Cells were selected with puromycin. Where indicated, cells were fixed,

permeabilized and stained intracellularly for IRG1 (Abcam), SDHA (Abcam) and IDH (Abcam). Cells were analyzed by flow cytometry for these markers. In these cells, succinate dehydrogenase activity was analyzed in total protein extracts (Abcam). When indicated, non-corrected CFBE cells were infected with an MOI of 20,000 with a PTEN-GFP or a GFP-coding adenovirus for 48h. Then, cells were washed and stained for mitochondrial membrane potential and mitochondrial ROS. The GFP^+ population (cells expressing GFP or PTEN-GFP) was analyzed for mitochondrial ROS production. The non-transduced population (GFP^-) was also analyzed for mitochondrial ROS.

Confocal Microscopy - 16HBE cells (epithelial cells) were grown on sterile-coverslips in 12-well plates until reaching 50-60% confluence. Cells were fixed for 20min with 2% PFA at 4°C, washed with PBS and permeabilized at RT with 0.05% Saponin-PBS-2% FBS for 15min. Coverslips were passed to a humid-chamber and stained with 50uL containing 1/200 goat anti-human PTEN, mouse anti-human CFTR and rabbit anti-human Tomm20. Antibodies were resuspended in permeabilization buffer. Cells were incubated at 4°C overnight. Cells were washed with 200uL PBS and 50uL of mix containing 1/500 of donkey anti-goat AF488 H+L, donkey anti-mouse AF647 H+L and donkey anti-rabbit AF546 H+L (Life technologies). Antibodies were resuspended in permeabilization buffer. Staining was for 3h. Cells were washed with PBS, dried and mounted with DAPI-containing anti-Fade Pro-long. Cells were analyzed in a Nikon Confocal Microscopy 60x magnification. Data analyses for colocalization were performed with Fiji software.

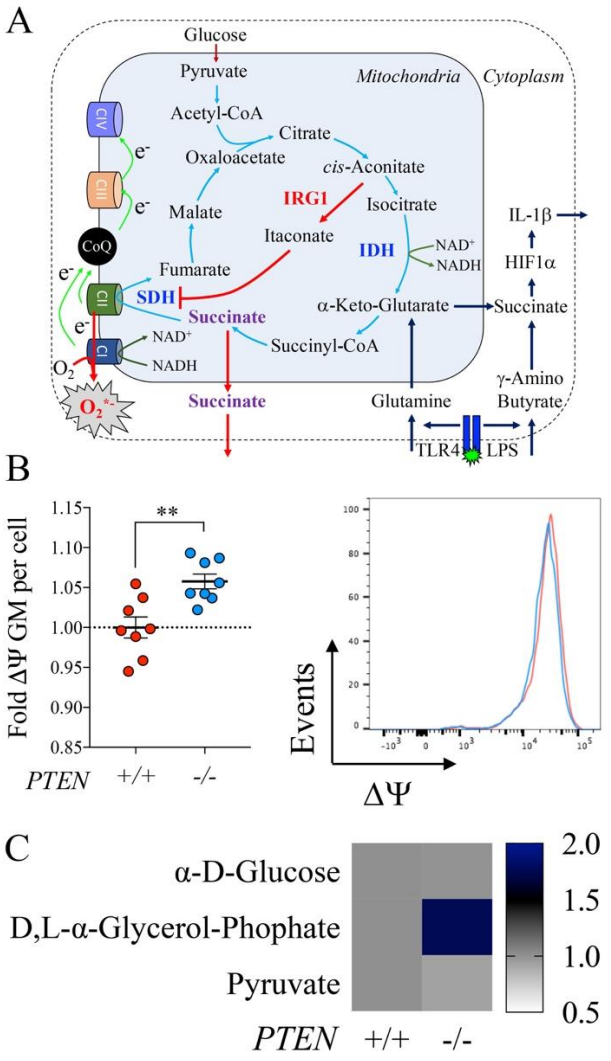

Fig. S1. PTEN affects mitochondrial membrane potential and assimilation of metabolites. A) Scheme showing regulation of mitochondrial TCA cycle. SDH-mediated ROS (O_2^{*-}) production is regulated by IRG1. SDH and IDH are pro-oxidant and highlighted in blue. IRG1 is anti-oxidant and highlighted in red lines. Light blue arrows show the normal course of reaction through the TCA cycle. Bottom red arrows show succinate secreted from cells. **B)** *PTEN*$^{+/+}$ and *PTEN*$^{-/-}$ HTC116 cells were stained for mitochondrial membrane potential ($\Delta\Psi$) and analyzed by flow cytometry. **C)** *PTEN*$^{+/+}$ and *PTEN*$^{-/-}$ HTC116 cells were placed in 96-well PM-M1 Biolog plates. The assimilation of specific single metabolites was analyzed and quantified. Data are shown as mean +/- SEM of three independent experiments. Student's t-test, ** P<0.01.

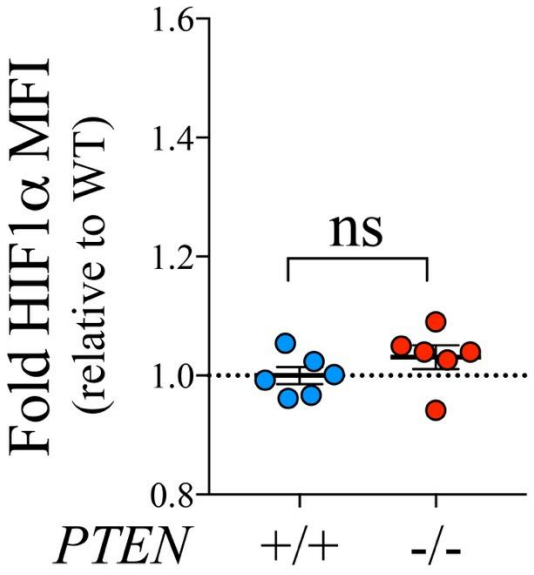

Fig. S2. HIF1α is not increased in PTEN-null cells. *PTEN*$^{+/+}$ and *PTEN*$^{-/-}$ HTC116 cells were stained for intracellular hypoxia-induced factor 1α (HIF1α) and analyzed by flow cytometry. Data are shown as mean +/- SEM of three independent experiments, with 6 replicates in total. Student's t-test, *ns*: non-significant.

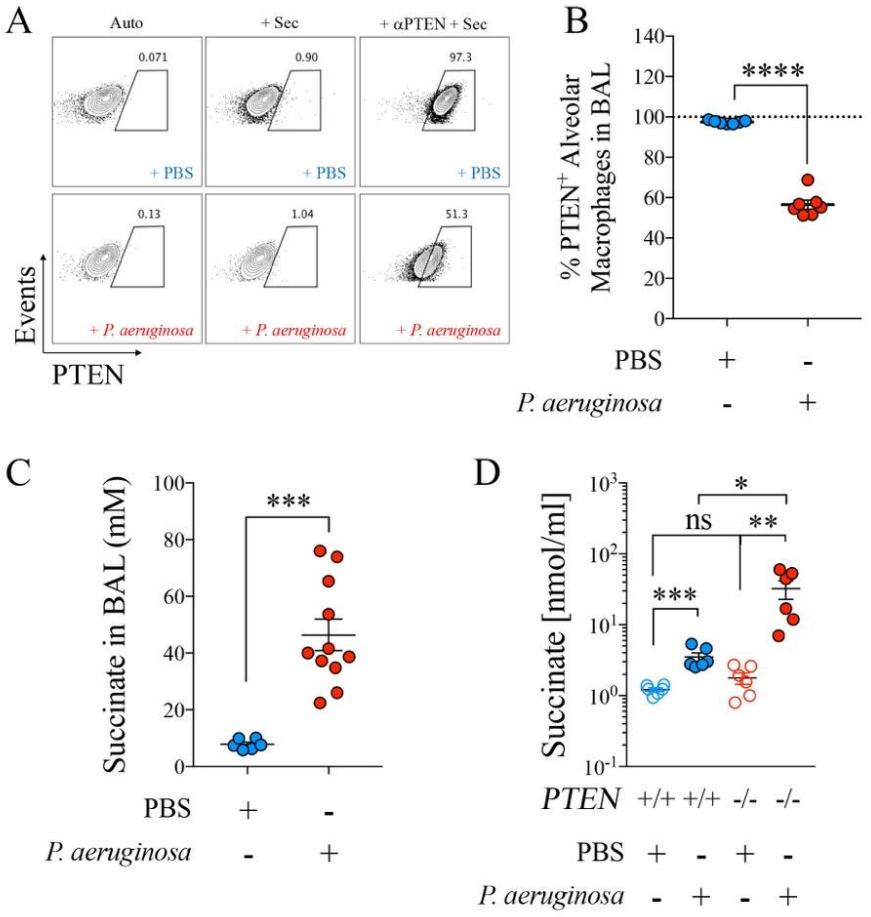

Fig. S3. PTEN regulates succinate secretion during infection with *P. aeruginosa*. A) WT mice were intranasally infected with 10^7 CFU of *P. aeruginosa* (PAO1). 24h later, alveolar macrophages recovered from BAL were intracellularly stained for PTEN. Auto = autofluorescence (non-stained alveolar macrophages); Sec = alveolar macrophages treated only with secondary antibody; Prim + Sec = alveolar macrophages treated with anti-PTEN and secondary antibody. **B)** Percent $PTEN^+$ alveolar macrophages (from **A**) in BAL quantification. **C)** From mice infected in **A**, succinate was quantified in BAL. **D)** $PTEN^{+/+}$ and $PTEN^{-/-}$ DLD1 epithelial human cells were left untreated or infected with *P. aeruginosa* for 2h, washed, treated with gentamicin overnight and then succinate was quantified in the supernatants. For **A-C**, data is from two independent experiments with 8-11 mice in total. Data in **D** is from 2 independent experiments with 6 replicates in total. Data is shown as mean +/- SEM. Student's t-test or one-way ANOVA, **** P<0.0001; *** P<0.001; ** P<0.01; * P <0.05; ns: non-significant.

A

Healthy Patients (*CFTR/CFTR*)	Cystic Fibrosis (*CFTR/CFTR*)
WT/WT	G542X/W1282X
	ΔF508/2789+5G>A
	ΔF508/R1066C
	ΔF508/R1066C
	ΔF508/3849+10Kb
	ΔF508/Q220X
	3849 + 10Kb/3849 + 10Kb
	3772-26A>G/3772-26A>G
	WT/W1282X
	ΔF508/3849+10Kbc>TY

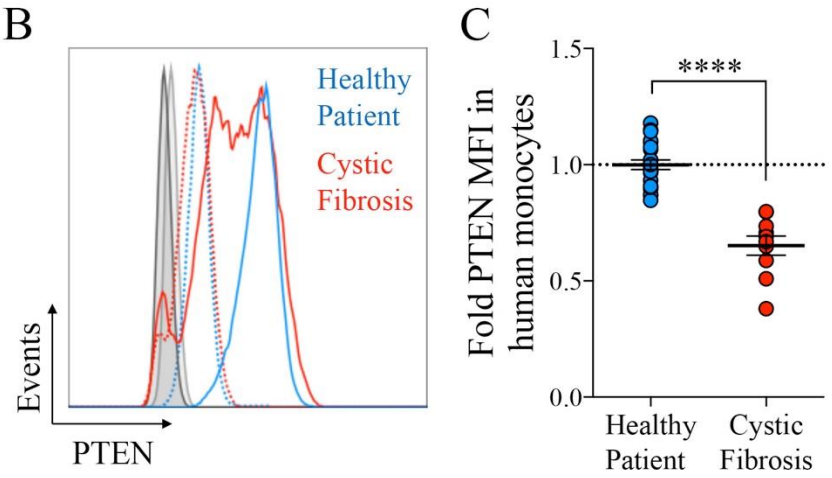

Fig. S4. PTEN is reduced in patients with *CFTR* mutations. A) *CFTR* genotype of patients with CF (n=10) and several healthy volunteers analyzed for PTEN expression. All healthy controls were not suffering from CF (*CFTR* WT/WT). **B)** Representative histogram for PBMCs from the blood of healthy and CF patients. PBMCs were purified and intracellularly stained for PTEN and analyzed by flow cytometry. **C)** Quantification of PTEN MFI in PBMCs obtained from healthy volunteers (n=24) and CF patients (n=10). Each point represents one patient. Data is shown as mean +/- SEM. Student's t-test, **** *P*<0.0001.

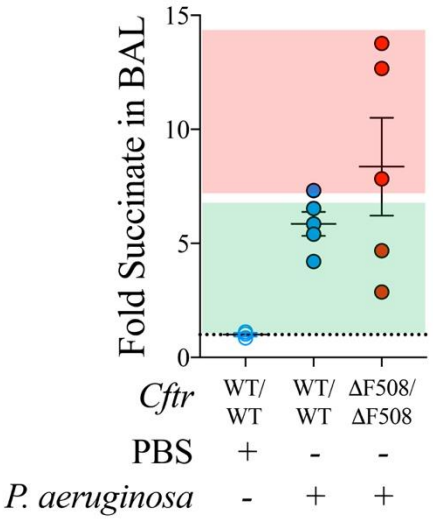

Fig. S5. Succinate in BAL is increased in *P. aeruginosa*–infected CFTR$^{\Delta F508/\Delta F508}$ mice. WT (n=5) and CFTR $^{\Delta F508/\Delta F508}$ mice (gut-corrected) (n=5) were challenged intranasally with 10^7 *P. aeruginosa* CFU/animal, and 24h later succinate was quantified in broncheoalveolar lavage.

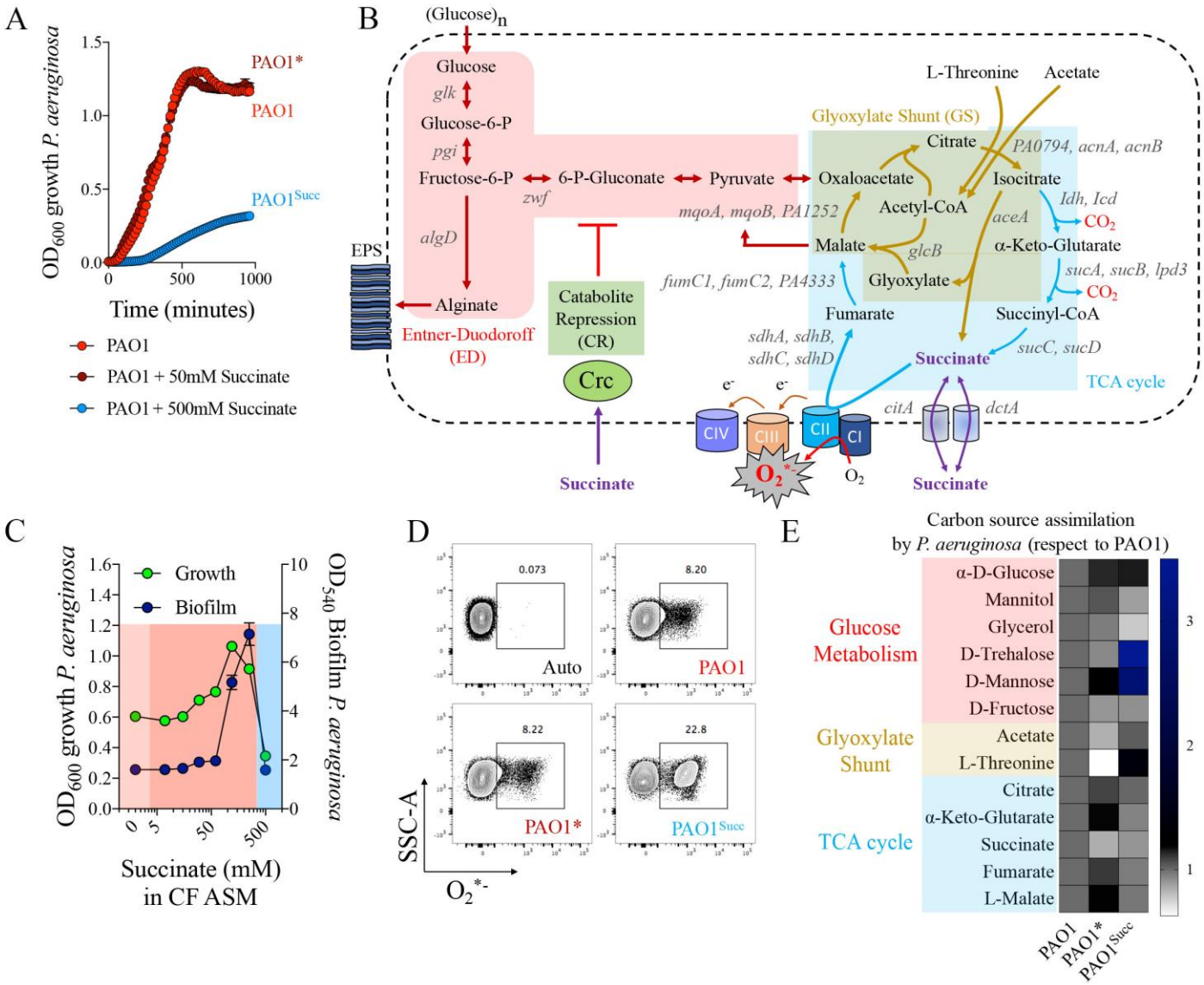

Fig. S6. High succinate induces oxidative stress and metabolic adaptation in *P. aeruginosa* in LB and CF sputum–like media. A) Growth curves of WT *P. aeruginosa* (PAO1) in LB without succinate (PAO1), 50mM succinate (PAO1*) or 500mM succinate ($PAO1^{Succ}$). **B**) *P. aeruginosa* metabolic activity. SDH-mediated O_2^{*-} generation by succinate is bypassed by using the glyoxylate shunt, which feeds alginate production through gluconeogenesis. **C**) PAO1 was grown overnight in increasing concentrations of succinate in CF artificial sputum media (CF ASM) and growth and biofilm was quantified. **D**) PAO1 was incubated either in LB, LB + 50mM or LB + 500mM succinate for 2h. Then, bacteria were washed and stained with MytoSox to detect anion superoxide (O_2^-) by flow cytometry. **E**) After overnight growth in CF sputum-like media supplemented without or +50mM/500mM succinate, analysis of bacterial assimilation was performed. Data in **A**, B and **D** represents two independent experiments and in **C**, three independent experiments.

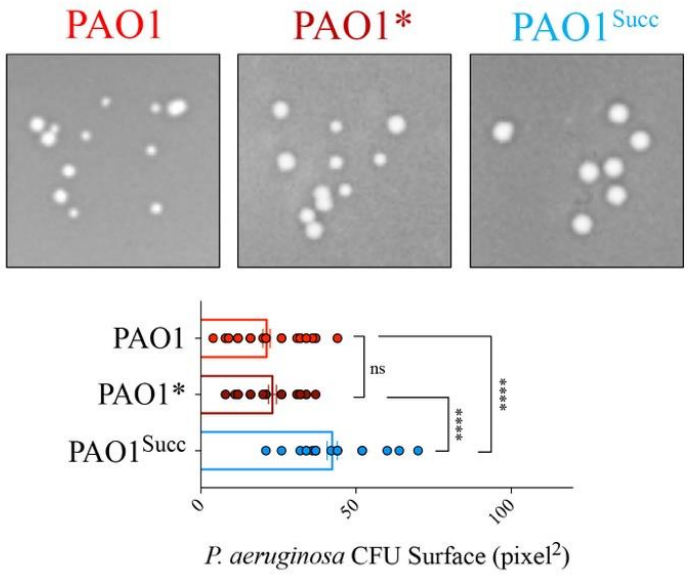

Fig. S7. Succinate-stressed WT *P. aeruginosa* produce larger colonies in succinate-free LB agar plates. WT *P. aeruginosa* (PAO1) were stressed overnight either in 50mM (PAO1*) or 500mM (PAO1^{Succ}) succinate or not (PAO1), washed and plated on succinate-free LB agar plates. After 24h incubation at 37°C, colony sizes were measured. The bottom graph displays the average CFU sizes for the different PAO1. Data are from 3 independent experiments. Data are shown as mean +/- SEM. Data were analyzed by one-way ANOVA. *****: P*<0.0001; ns: non-significant.

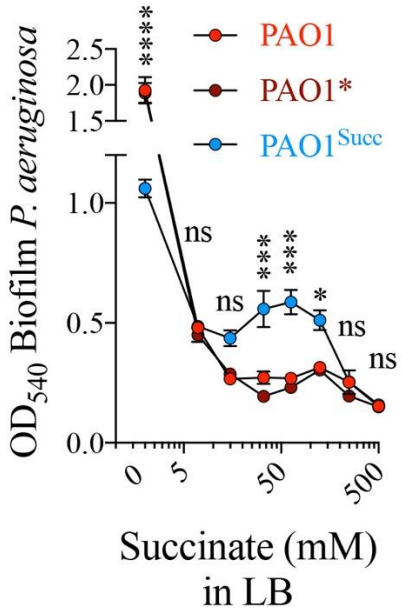

Fig. S8. Succinate-stressed *P. aeruginosa* biofilms are more tolerant to succinate. PAO1 was incubated overnight either in LB (PAO1), LB + 50mM succinate (PAO1*) or LB + 500mM succinate ($PAO1^{Succ}$). Then, bacteria were washed and re-incubated in increased concentrations of succinate overnight. Biofilms production was measured. Data were analyzed by two-way ANOVA and represent three independent experiments. Data are shown as mean +/- SEM. *****: P*<0.0001; ****: P*<0.001; ***: P*<0.01*; *: P*<0.05; ns: non-significant.

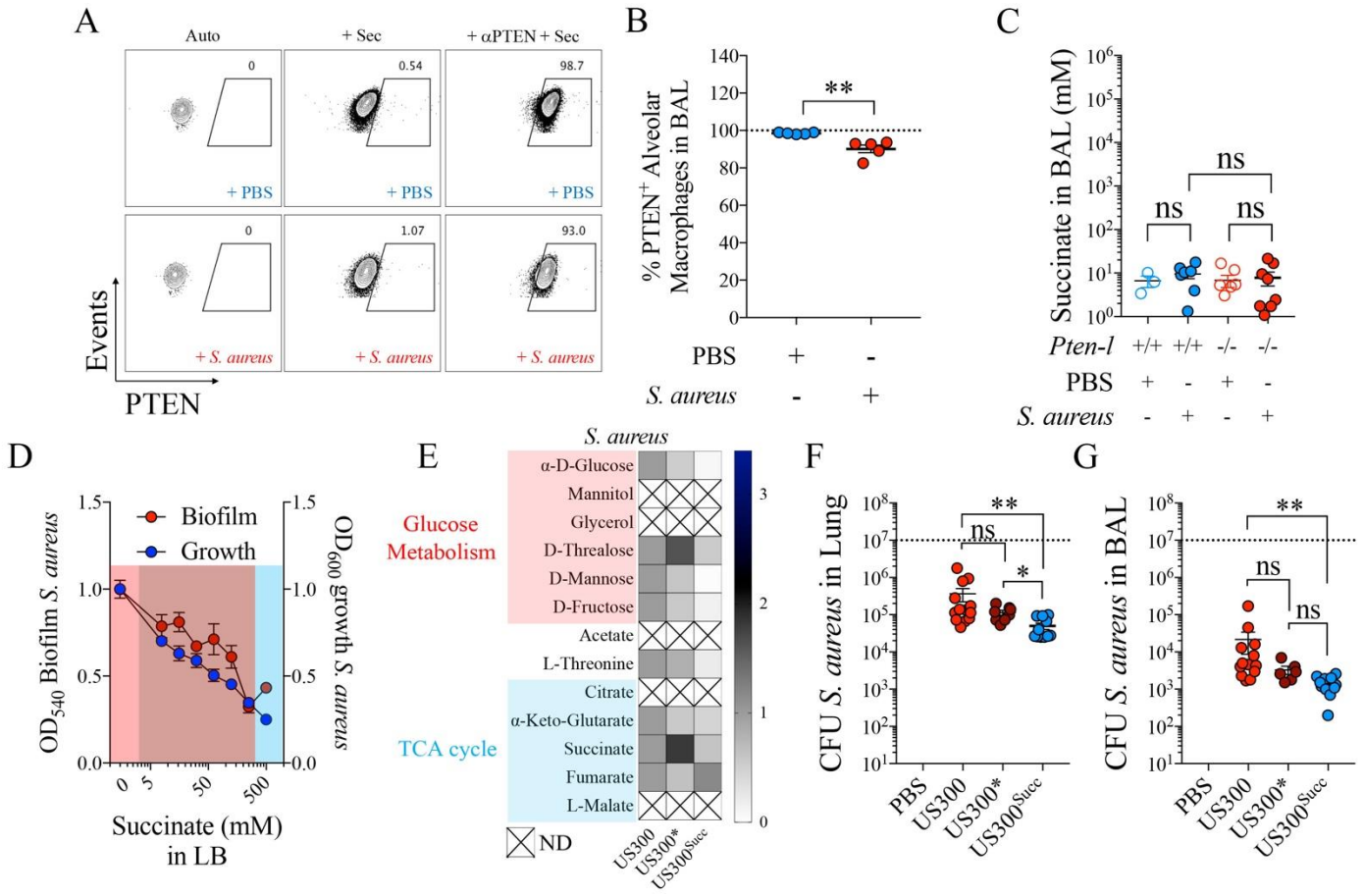

Fig. S9. The PTEN-succinate axis does not regulate *S. aureus* adaptation to the airway. A) Alveolar macrophages from PBS-treated or *S. aureus* (MRSA)-infected BAL were intracellularly stained for PTEN and analyzed by flow cytometry. **B**) Percentage of PTEN^{+} alveolar macrophages from untreated or *S. aureus*-infected mice from **A**. **C**) BAL succinate concentration from WT or *Ptenl*-deficient mice treated 24h intranasally either with PBS or *S. aureus*. **D**) *S. aureus* were grown overnight in LB containing increasing concentrations of succinate. Growth and biofilm were quantified. **E**) *S. aureus* growth overnight in LB (US300), LB + 50mM succinate (US300*) or LB + 500mM succinate (US300^{Succ}) were plated on PM1 Biolog plates to measure metabolite consumption. ND: non-detected. **F-G**) Mice were intranasally infected with 107 CFU of USA300, USA300* or USA300^{Succ}. 24h later, mice were sacrificed and bacterial loads were quantified in lung and BAL. Data represents three independent experiments. Data is shown as mean +/- SEM. A, Student's t-test; **C**, **F** and **G**, one-way ANOVA. **: P*<0.05*; **: P*<0.01*; ***: P*<0.001*; ****: P*<0.0001; ns: non-significant.

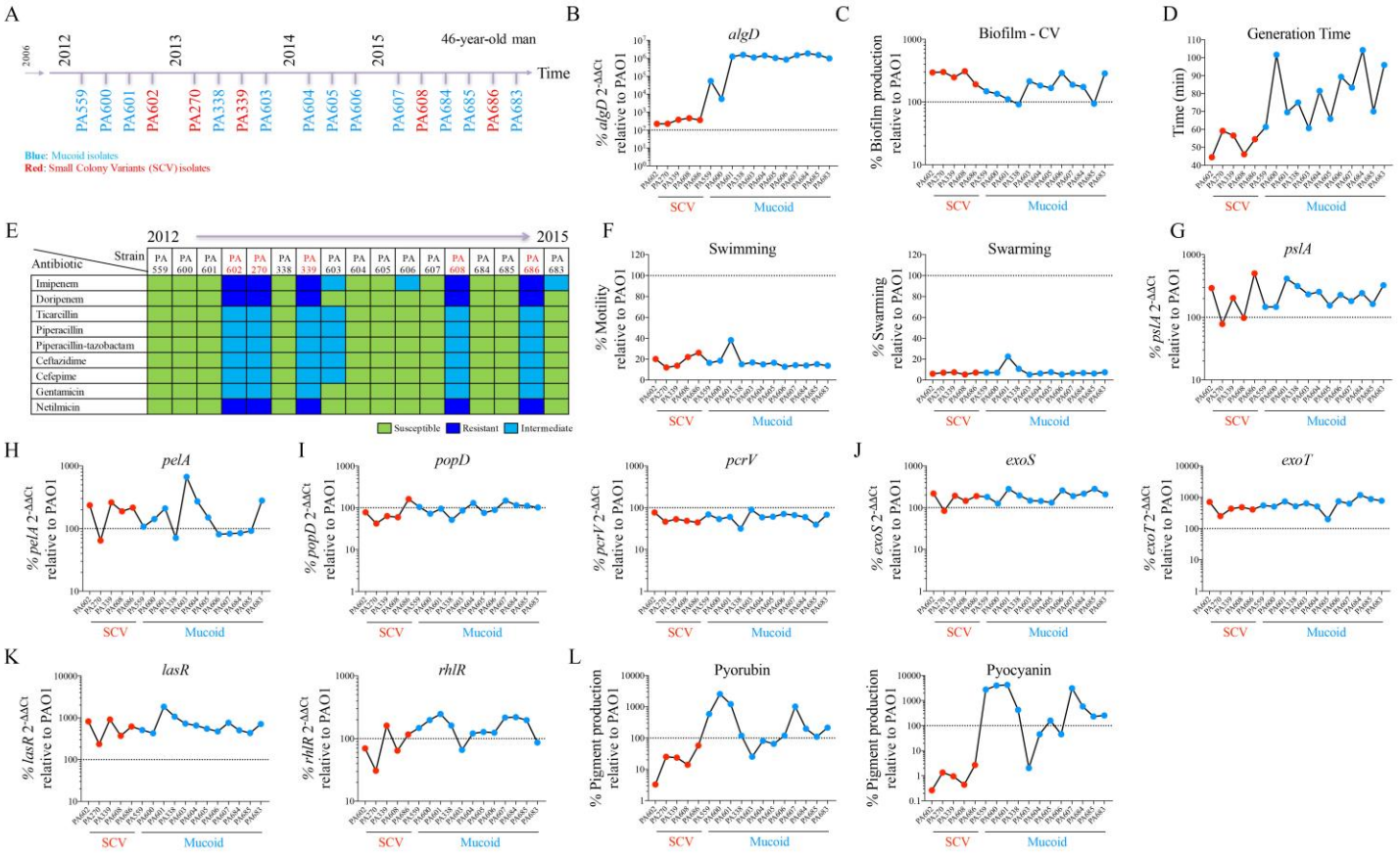

Fig. S10. Phenotypic characterization of *P. aeruginosa* strains recovered over 4 years from the CF airway. A) From a chronically infected, 46-year-old CF patient, 17 *P. aeruginosa* isolates were collected from 2012 to 2015. Based on plate morphology, strains were classified as small colony variants (SCV) or mucoid. **B)** *AlgD* expression profile for each isolate and compared with PAO1. **C)** Capacity of biofilm production for each isolate through crystal violet (CV). **D)** Generation time measured for each isolate. **E)** Antibiogram for each isolate against different antibiotics used in CF clinics. **F)** Swimming and swarming motility profile for each isolate. **G-H)** Biofilm *pslA* and *pelA* genes expression profile for each isolate. **I)** *popD* and *pcrV* expression profile, components of the type-three secretion system (TTSS) needle, for each isolate. **J)** *exoT* and *exoS* expression profile, virulence factors secreted with the TTSS needle, for each isolate. **K)** *lasR* and *rhlR* expression profile, components of the quorum system (QS) and pro-biofilm, for each isolate. **L)** Pyorubin and pyocyanin pigment production profile. Data is shown as the mean of three independent measurements for each strain.

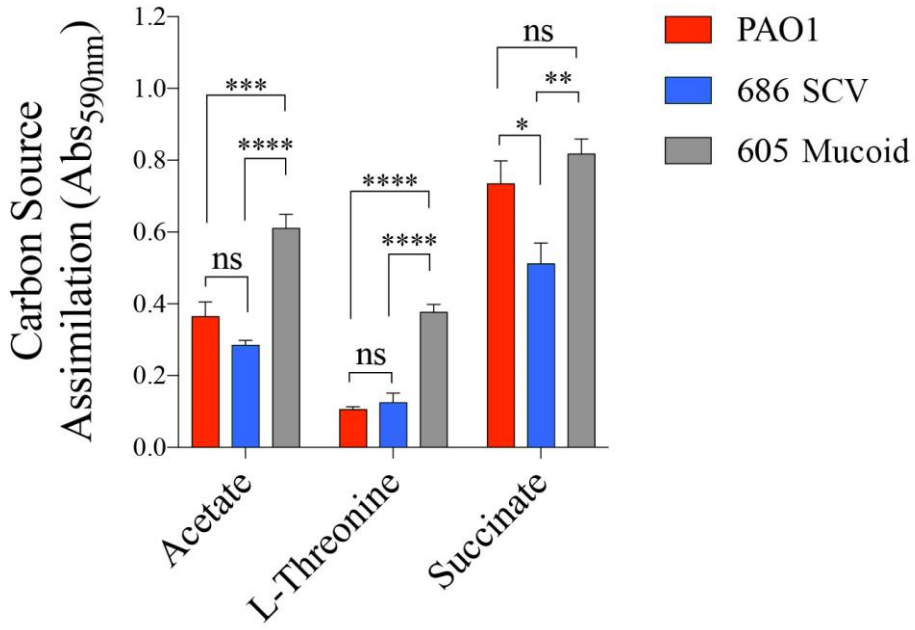

Fig. S11. Mucoid and SCV CF isolates have differential preference for succinate, acetate, and l-threonine compared with PAO1. PAO1, SCV 686, or mucoid 605 were inoculated in a 96-well Biolog PM1 plate. Nutrient utilization was followed for 48h-72h by absorbance at OD_{590nm}. Data represent five independent experiments and values are shown as mean +/- SEM. One-way ANOVA.,****: *P*<0.0001; ***: *P*<0.001; **: *P*<0.01; *: *P*<0.05; ns: non-significant.

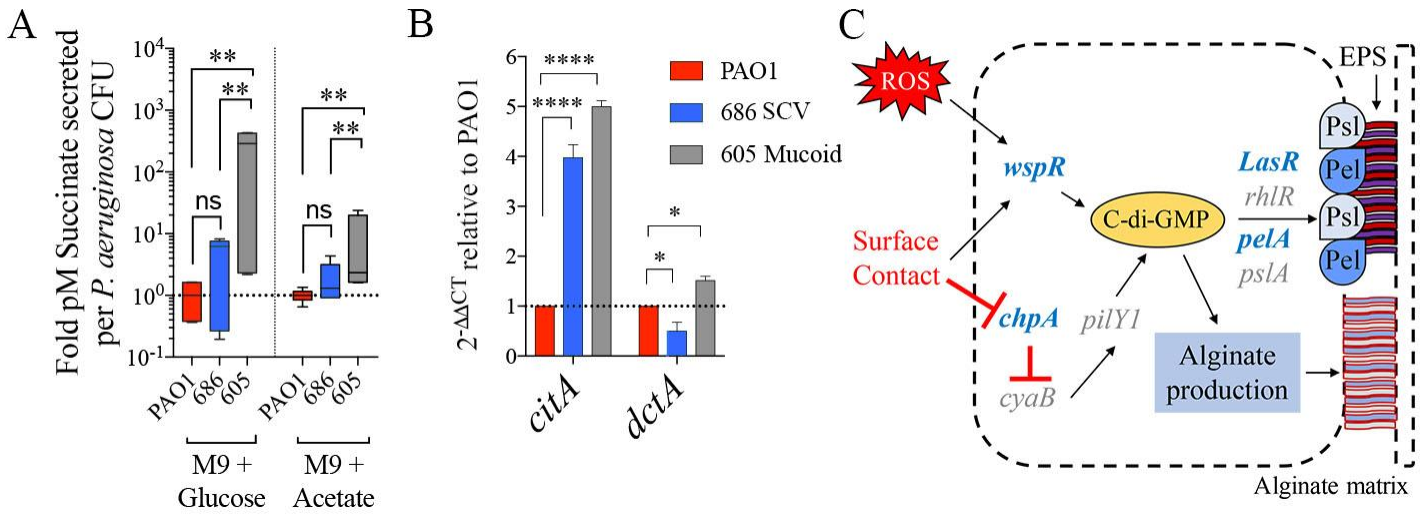

Fig. S12. CF-adapted *P. aeruginosa* excrete succinate. A) PAO1, 686 or 605 strains were incubated overnight in minimal media (M9) supplemented either with α-D-glucose or acetate and succinate accumulation was quantified in the supernatant. Succinate concentrations were standardized against the amount of CFU per sample. **B)** Fold change in mRNA expression for the citrate/succinate antiporter *citA* and *dctA* C4 dicarboxylate transporter for SCV 686 and mucoid 605 relative to PAO1. These genes had the same nonsynonymous mutations in all CF isolates. **C**) Diagram of pathways regulating c-di-GMP and biofilm production in *P. aeruginosa.* Data represent 3 independent experiments and values are shown as mean +/- SEM. Data were analyzed by one-way ANOVA. ****: *P*<0.0001; **: *P*<0.01; *: *P*<0.05; ns: non-significant.

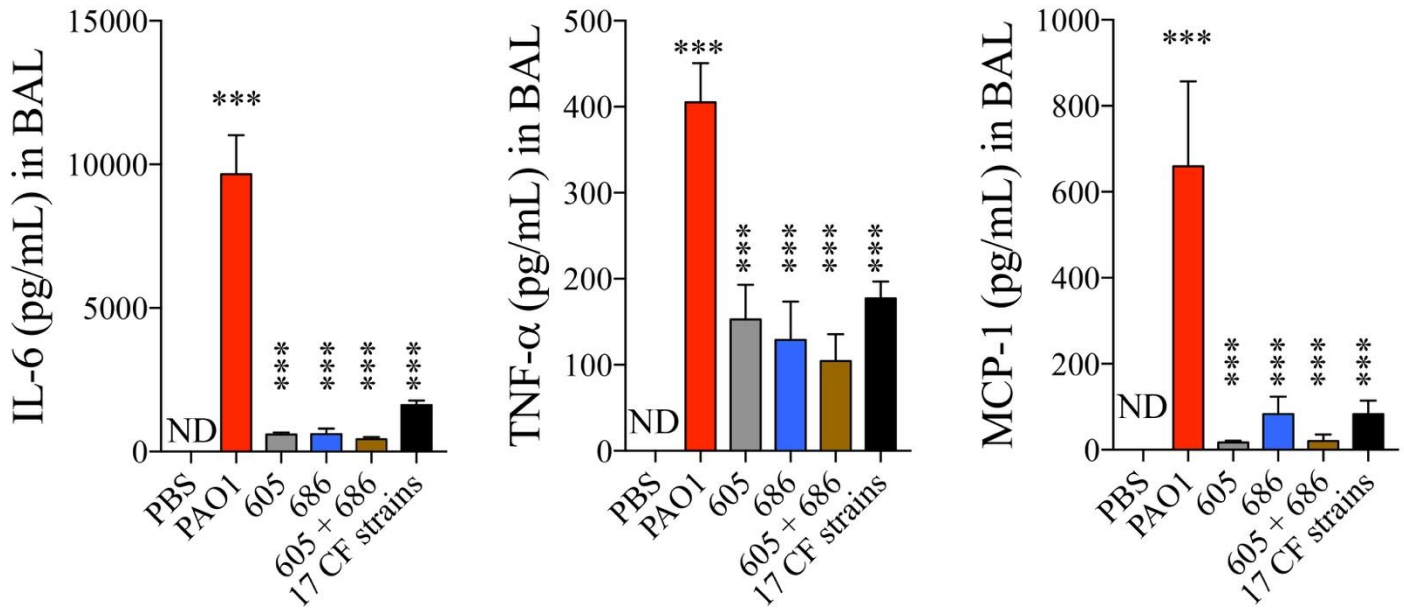

Fig. S13. Host-adapted *P. aeruginosa* induce less secretion of proinflammatory cytokines in the airway. C57bl/6 mice were intranasally infected with 1 x 10^7 CFU of either laboratory strain PAO1, 1 x 10^7 CFU of SCV variant 686, 1 x 10^7 CFU of mucoid strain 605, a total 1 x 10^7 CFU mix of both 686 + 605 (5 x 10^6 of each) or with a total 1 x 10^7 CFU mix of all 17 isolates (equal parts at 5.9 x 10^5 for each). IL-6, TNF-α and MCP-1 concentration in broncheoalveolar lavage was analyzed by ELISA. Data is from two independent experiments each with n=3 (6 mice in total) and shown as mean +/- SEM. One-way ANOVA. ****: *P*<0.0001; ***: *P*<0.001. **: *P*<0.01; *: *P*<0.05; ns: non-significant. ND: non-detected.

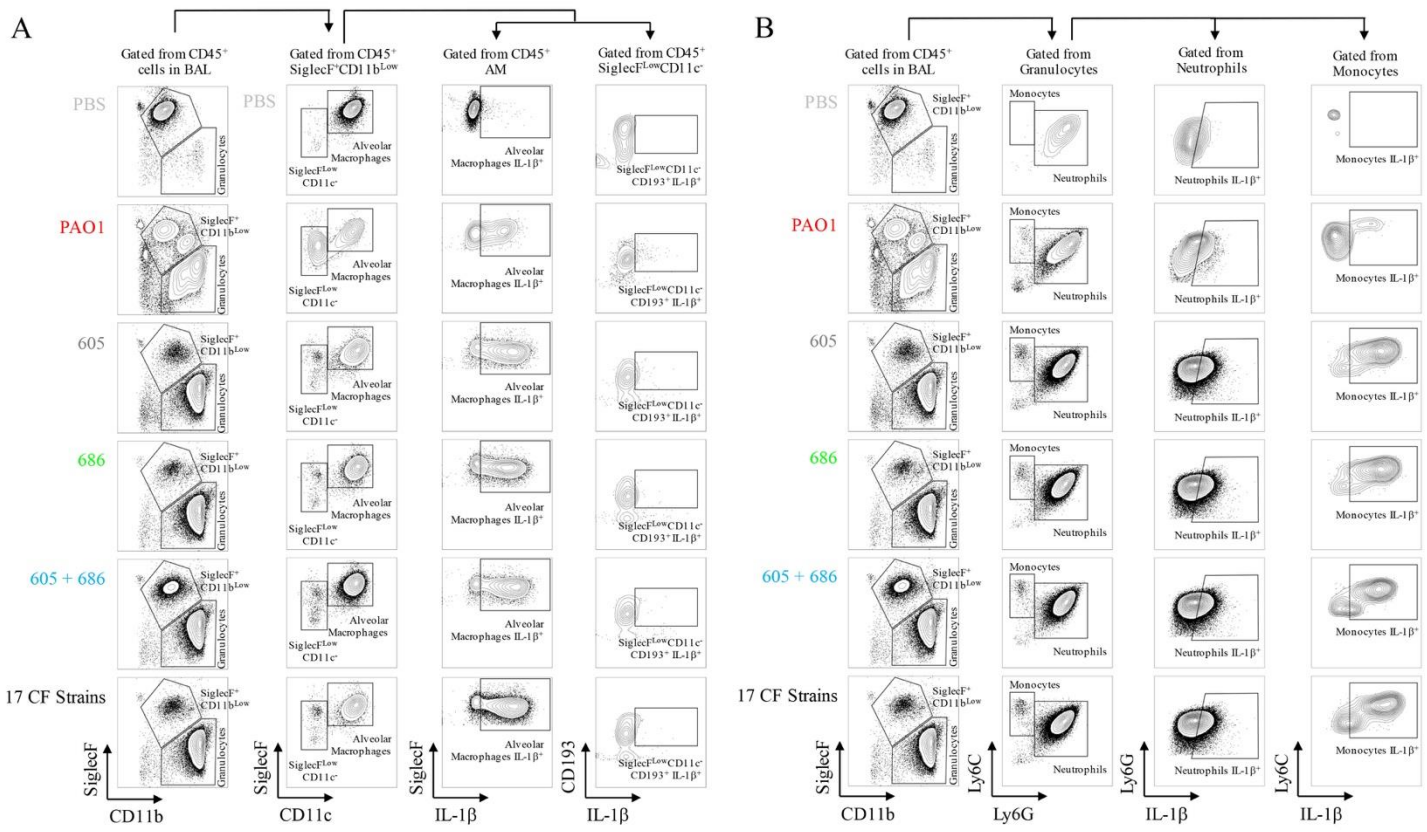

Fig. S14. IL-1β induced by metabolically adapted *P. aeruginosa* isolates is not trapped inside neutrophils. C57bl/6 mice were intranasally infected with either 1 x 10^7 CFU of laboratory strain PAO1, 1 x 10^7 CFU of SCV variant 686, 1 x 10^7 CFU of mucoid strain 605, a total 1 x 10^7 CFU mix of both 686 + 605 (5 x 10^6 of each), or with a total 1 x 10^7 CFU mix of all 17 isolates (equal parts – 5.9 x 10^5 each). Intracellular staining for pro-IL-1β was performed in (**A**) alveolar $CD11c^+SiglecF^+$ macrophages and $CD11c^-SiglecF^{low}CD193^+$ eosinophils, and (**B**) monocytes $Ly6G^-Ly6C^+$ and $Ly6G^+Ly6C^+$ neutrophils. Gating strategy is shown. Data shown is representative of two independent experiments, n=3 for each experiment (6 mice in total).

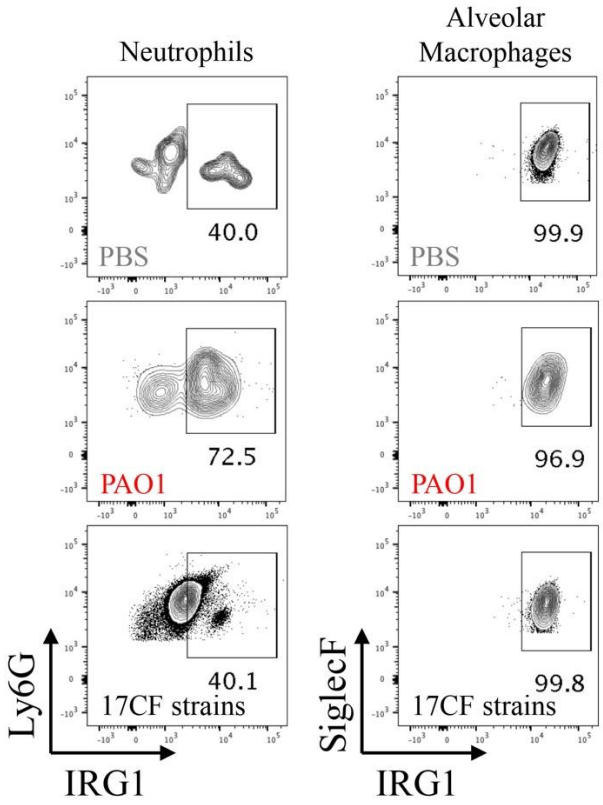

Fig. S15. IRG1 expression by resident alveolar macrophages and neutrophils in *P. aeruginosa*–infected mice. C57bl/6 mice were intranasally treated with PBS or infected with 1 x 10^7 CFU of PAO1 or with a total 1 x 10^7 CFU mix of all 17 isolates (equal parts – 5.9 x 10^5 for each). Intracellular staining for IRG1 was performed in $Ly6G^+Ly6C^+$ recruited neutrophils (left) and alveolar $CD11c^+SiglecF^+$ macrophages (right). Data shown are representative of two independent experiments, with 6 mice in total.

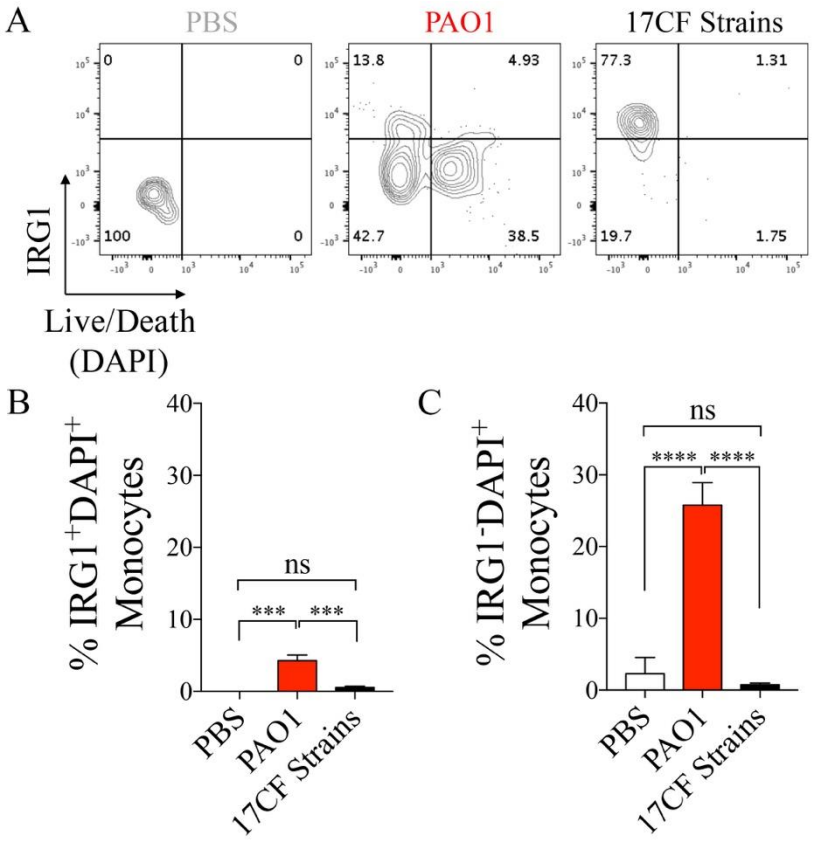

Fig. S16. Clinical isolates of *P. aeruginosa* do not induce cell death in IRG1$^+$ BAL monocytes. C57bl/6 mice were intranasally infected with 1 x 10^7 CFU of laboratory strain PAO1 or a total mix of all 17 isolates (equal parts – 5.9 x 10^5 for each). **A)** BAL monocytes were stained for IRG1 and cell viability (DAPI, cell membrane permeabilization). **B-C**) Percentage of IRG$^+$DAPI$^+$ and IRG$^-$DAPI$^+$ monocytes. Data shown is representative of two independent experiments, n=3 for each experiment (6 mice in total). Graphs are shown as mean +/- SEM. Data were analyzed by one-way ANOVA. ****: *P*<0.0001; ns: non-significant.

Table S1. *CFTR* genotype and *P. aeruginosa* airway abundance in healthy patients and patients with CF. Healthy volunteers (n=8) and patients with CF (n=25) were tested for *P. aeruginosa* positive or negative culture and *P. aeruginosa* 16S abundance. When possible, the *CFTR* genotype was obtained from patients with CF.

Patient	PA culture	% PA 16S Abundance	*CFTR* Allele 1	*CFTR* Allele 2
Healthy Patient 1	Negative	0.99378882	WT	WT
Healthy Patient 2	Negative	0	WT	WT
Healthy Patient 3	Negative	0	WT	WT
Healthy Patient 4	Negative	0.894409938	WT	WT
Healthy Patient 5	Negative	0	WT	WT
Healthy Patient 7	Negative	0	WT	WT
Healthy Patient 8	Negative	0	WT	WT
CF Patient 1	Negative	0	Undetermined	Undetermined
CF Patient 2	Negative	5.639751553	Undetermined	Undetermined
CF Patient 3	Negative	0	Undetermined	Undetermined
CF Patient 4	Negative	0	Undetermined	Undetermined
CF Patient 5	Negative	0.006056935	F508del	G542X
CF Patient 6	Negative	0.079997539	Undetermined	Undetermined
CF Patient 7	Negative	0.015895724	Undetermined	Undetermined
CF Patient 8	Negative	0.012759578	W1282X	W1282X
CF Patient 9	Negative	0.029135407	Undetermined	Undetermined
CF Patient 10	Negative	0.968961714	Undetermined	Undetermined
CF Patient 11	Positive	7.164387751	Undetermined	Undetermined
CF Patient 12	Negative	0	F508del	F508del
CF Patient 13	Negative	0.178448868	F508del	F508del
CF Patient 14	Negative	0.023296835	F508del	Y849X
CF Patient 15	Negative	0.24152991	F508del	F508del
CF Patient 16	Negative	0.04165089	F508del	PY849X
CF Patient 17	Negative	0.032664794	Undetermined	Undetermined
CF Patient 18	Negative	0.023824479	Undetermined	Undetermined
CF Patient 19	Positive	10.63455303	3120+1G->A	3120+1G->A
CF Patient 20	Negative	0.008981498	Undetermined	Undetermined
CF Patient 21	Positive	97.0713525	Undetermined	Undetermined
CF Patient 22	Negative	0.013170459	Undetermined	Undetermined
CF Patient 23	Negative	0.696069256	Undetermined	Undetermined
CF Patient 24	Negative	Undetermined	Undetermined	Undetermined
CF Patient 25	Negative	Undetermined	Undetermined	Undetermined

Table S2. Pathoadaptive mutations conserved in all 17 CF *P. aeruginosa*. Number of synonymous (SM) and non-synonymous mutations (NSM) found in different genes in all 17 *P. aeruginosa* isolates respect with control PAO1. Changes found were equally conserved in all *P. aeruginosa* isolates.

Pathway	Genes (NSM/SM)
Antibiotic resistance	PA2491 (*mexS*) (1/0) *mexT* (1/6) *ampD* (1/2) *pmrA* (1/4) *gyrB* (1/7) *parE* (1/16) *mpl* (2/5)
Transport	PA0313 *(yecS)* (1/0)
Mucoidy and biofilm	*morA* (1/11) PA0861(*rbdA*) (3/16) *retS* (1/9) *alg8EXIWP* (1/1, 2/8, 1/6, 3/2, 1/0, 2/3*)* *muck (2/11)*
c-di-GMP signaling pathway and biofilm	*wspACFR* (1/3, 1/7, 1/2, 1/2) *pslCFHIM* (4/1, 2/1, 1/1, 1/0, 2/4) *pelABDEF* (4/11, 5/5, 5/15, 4/2, 1/3) *chpAC* (6/19, 2/4)
Quorum sensing and biofilm	*lasR* (1/0) *rhlACGI* (1/3, 2/3, 3/0, 3/7) *pqsDE* (1/4, 1/0) *phnA* (2/1)
Flagella assembly	*flgJL* (1/1,13/22) *fliC* (22/33) PA1095 (3/2) *fliE* (1/1) PA1103 (1/1) PA1441 (3/11) *flhF* (4/1) *motDCA* (1/2,1/0,3/14) PA3352 (1/4)
Motility	*pil BSQ* (19/53,3/9,15/36) *rpoN* (3/7) *cup C1C2C3A1A4B6B5B4B3B2B1* (10/21, 7/14, 10/36, 1/1, 1/6 , 8/4, 16/25, 4/3, 3/16, 3/0, 10/4)
Glucose uptake pathway	*glk* (1/4) *fruK* (*fpk*) (3/9)
Entner-Doudoroff (ED) Pathway	*zwf* (1/4) PA2261 (*kgk*) (1/1) PA2263 (*kgr*) (2/1)
Glyoxylate Shunt (GS)	*aceA* (1/1) *glcB* (1/0)
TCA cycle	PA0794 (2/8) *idh* (3/3) *sucA* (1/6) *fumC1* (1/1)
Metabolism (Others)	*aceF* (2/1) *napFDA* (1/0, 1/2, 1/4) *norC* (1/0) *narK1K2I* (1/9, 1/4, 1/3) *nirFJN* (2/1, 3/0, 1/3) PA0521 (1/0)
TTSS (PA1690-1725)	PA1700 (1/3) *pcrDV* (2/18, 5/5) *popBD* (2/2, 1/2) *exsCD* (1/3, 1/0) *pscBCDEILPQTU* (1/2, 1/5, 1/3, 2/1, 1/0, 2/5, 8/2, 4/4, 1/4, 1/4)
TTSS translocated effectors	*exoYT* (2/3, 4/11)
Gac/Rsm pathway & T6SS (Hcp1 secretion island I)	*ladS* (3/8) PA0078/80/82/84/88 (1/2, 2/0, 4/6, 1/2, 2/2) *icmF1* (1/6) *clpV1* (3/7) *vgrG1* (1/0)
Phenazine biosynthesis	*phzB2A2A1S* (1/9,1/1,1/0,3/16)
Tad locus	*flp* (2/4) *rcpC* (2/2)

	tadG (1/4) PA4294(*tadF*) (1/6) *pprAB* (9/46, 3/3) *fppA* (2/2)
Iron acquisition	*pvdQRTPNOEDJLG* (2/20,1/5, 3/9, 16/101, 2/12, 9/9, 1/0, 16/34, 4/16,18/63, 1/3)

Table S3. Accession numbers for *P. aeruginosa* isolates.

Isolate	SRA_accession	Study	Bioproject_accession	Biosample_accession	Genome_accession
Pa270	SRR8775051	SRP189252	PRJNA528628	SAMN11241333	SPIS00000000
Pa338	SRR8775050	SRP189252	PRJNA528628	SAMN11241334	SPIR00000000
Pa339	SRR8775058	SRP189252	PRJNA528628	SAMN11241335	SPIQ00000000
Pa599	SRR8775057	SRP189252	PRJNA528628	SAMN11241336	SPIP00000000
Pa600	SRR8775065	SRP189252	PRJNA528628	SAMN11241337	SPIO00000000
Pa601	SRR8775054	SRP189252	PRJNA528628	SAMN11241338	SPIN00000000
Pa602	SRR8775055	SRP189252	PRJNA528628	SAMN11241339	SPIM00000000
Pa603	SRR8775053	SRP189252	PRJNA528628	SAMN11241340	SPIL00000000
Pa604	SRR8775056	SRP189252	PRJNA528628	SAMN11241341	SPIK00000000
Pa605	SRR8775052	SRP189252	PRJNA528628	SAMN11241342	SPIJ00000000
Pa606	SRR8775060	SRP189252	PRJNA528628	SAMN11241343	SPII00000000
Pa607	SRR8775059	SRP189252	PRJNA528628	SAMN11241344	SPIH00000000
Pa608	SRR8775062	SRP189252	PRJNA528628	SAMN11241345	SPIG00000000
Pa683	SRR8775061	SRP189252	PRJNA528628	SAMN11241346	SPIF00000000
Pa684	SRR8775064	SRP189252	PRJNA528628	SAMN11241347	SPIE00000000
Pa685	SRR8775063	SRP189252	PRJNA528628	SAMN11241348	SPID00000000
Pa686	SRR8775066	SRP189252	PRJNA528628	SAMN11241349	SPIC00000000

Table S4. PAMPs found mutated in all CF *P. aeruginosa* isolates. Number of non-synonymous mutations found in all 17 CF *P. aeruginosa* isolates respect with PAO1. These genes belong to pathways that have been characterized as activators of the inflammasome. These data are plotted in Figure 5A.

Pathway	Genes	Non-synonymous mutations (NSM)
LPS biogenesis and function	PA0595; *osta (lptD)*	7
LPS biogenesis and function	PA5447; *wbpZ*	7
LPS biogenesis and function	PA4999; *waaL*	3
LPS biogenesis and function	PA5449; *wbpX*	2
LPS biogenesis and function	PA5452 *wbpW*	1
LPS biogenesis and function	PA5456	1
LPS biogenesis and function	PA5459	0
LPS biogenesis and function	PA5448; *wbpY*	0
LPS biogenesis and function	PA5450; *wzt*	0
LPS biogenesis and function	PA5451; *wzm*	0
LPS biogenesis and function	PA5453; *gmd*	0
LPS biogenesis and function	PA5454; *rmd*	0
LPS biogenesis and function	PA5455	0
LPS biogenesis and function	PA5457	0
LPS biogenesis and function	PA5458	0
Flagella biogenesis and function	PA1092; *fliC*	22
Flagella biogenesis and function	PA1087; *flgL*	13
Flagella biogenesis and function	PA4954; *motA*	3
Flagella biogenesis and function	PA1460; *motC*	1
Flagella biogenesis and function	PA1461; *motD*	1
Flagella biogenesis and function	PA1085; *flgJ*	1
Flagella biogenesis and function	PA1100; *fliE*	1
Flagella biogenesis and function	PA3526; *motY*	0
Flagella biogenesis and function	PA4953; *motB*	0
Flagella biogenesis and function	PA1077; *flgB*	0
Flagella biogenesis and function	PA1078; *flgC*	0
Flagella biogenesis and function	PA1079; *flgD*	0
Flagella biogenesis and function	PA1080; *flgE*	0
Flagella biogenesis and function	PA1081; *flgF*	0
Flagella biogenesis and function	PA1082; *flgG*	0
Flagella biogenesis and function	PA1083; *flgH*	0
Flagella biogenesis and function	PA1084; *flgI*	0
Flagella biogenesis and function	PA1084; *flgk*	0
T3SS assembly and secretion	PA1695; *pscP*	8
T3SS assembly and secretion	PA1706; *pcrV*	5
T3SS assembly and secretion	PA0044; *exoT*	4
T3SS assembly and secretion	PA1694; *pscQ*	4
T3SS assembly and secretion	PA1717; *pscD*	3
T3SS assembly and secretion	PA2191; *exoY*	2

T3SS assembly and secretion	PA1703; *pcrD*	2
T3SS assembly and secretion	PA1708; *popB*	2
T3SS assembly and secretion	PA1718; *pscE*	2
T3SS assembly and secretion	PA1725; *pscL*	2
T3SS assembly and secretion	PA1709; *popD*	1
T3SS assembly and secretion	PA1698; *popN*	1
T3SS assembly and secretion	PA1710; *exsC*	1
T3SS assembly and secretion	PA1714; *exsD*	1
T3SS assembly and secretion	PA1715; *pscB*	1
T3SS assembly and secretion	PA1716; *pscC*	1
T3SS assembly and secretion	PA1722; *pscI*	1
T3SS assembly and secretion	PA1691; *pscT*	1
T3SS assembly and secretion	PA1690; *pscU*	1
T3SS assembly and secretion	PA3841; *exoS*	0
T3SS assembly and secretion	PA1705; *pcrG*	0
T3SS assembly and secretion	PA1713; *exsA*	0
T3SS assembly and secretion	PA1712; *exsB*	0
T3SS assembly and secretion	PA1719; *pscF*	0
T3SS assembly and secretion	PA1720; *pscG*	0
T3SS assembly and secretion	PA1721; *pscH*	0
T3SS assembly and secretion	PA1723; *pscJ*	0
T3SS assembly and secretion	PA1724; *pscK*	0
T3SS assembly and secretion	PA1696; *pscO*	0
T3SS assembly and secretion	PA1693; *pscR*	0
Type IV Pilus biogenesis and function	PA4526; *pilB*	19
Type IV Pilus biogenesis and function	PA5040; *pilQ*	15
Type IV Pilus biogenesis and function	PA4525; *pilA*	0
Type IV Pilus biogenesis and function	PA4528; *pilD*	0
Type IV Pilus biogenesis and function	PA5041; *pilP*	0
Type IV Pilus biogenesis and function	PA5042; *pilO*	0
Type IV Pilus biogenesis and function	PA5043; *pilN*	0
Type IV Pilus biogenesis and function	PA5044; *pilM*	0

Table S5. Sequences of the primers used for qRT-PCR.

Gene	Primer name	Primer sequence (5'-3')	Reference
aceA	qaceA-F	ACAGCCCGTCCTTCAACTGG	This study
	qaceA-R	AGCTTGTTGCGGTCGTAGGC	This study
acnA	qacnA-F	GGCGAAGAAGGCAGTGGAGA	This study
	qacnA-R	CGACCAGATCGAAGCCGAGT	This study
acnB	qacnB-F	ATTCCTTTCCGCCATCGTCA	This study
	qacnB-R	ATGTTGTAGCCGCCCTGCAT	This study
citA	qcitA-F	CGTCGAGTTGTGGCTGTCGT	This study
	qcitA-R	CAGGCTGTAGGCGAGGGAGA	This study
crc	qcrc-F	TCCTTCCAACTGGACGGCTA	Janjua et al., 2012 (*45*)
	qcrc-R	AGCAGGGTGGCGATACTCAC	Janjua et al., 2012 (*45*)
dctA	qdctA-F	CTTCAACCTCGACGGCACCT	This study
	qdctA-R	CTTGGAAGCGATCAGCAGCA	This study
fruk	qfruK-F	AGCCGAACGTCGAGGAACTG	This study
	qfruK-R	CCCTGGGAAATCACCACCTG	This study
fumC1	qfumC1-F	CATGAACGCCAACGAAGTGG	This study
	qfumC1-R	CGGCACTGATGTGGATGGTC	This study
fumC2	qfumC2-F	CGCACATCGGCTACGACAAG	This study
	qfumC2-R	GAACTGCGCTTCGTCCAGGT	This study
glcB	qglcB-F	CGCCACTTCCTACAGCGTGA	This study
	qglcB-R	GCCCTGGAAGGCAAGGAACT	This study
glk	qglk-F	CTCGGTGGCGTCTACATTAC	This study
	qglk-R	GTAAGCGCCGCTAGTCTT	This study
glpD	qglpD-F	TTCGCCATCCCCTATCTGGA	This study
	qglpD-R	GCTGCTTGAAATGGGCGTTC	This study
idh	qidh-F	GGCGATGATCCGCAACTC	Tatke et al., 2015 (*21*)
	qidh-R	GCATTACCGCCTTGGTGTCT	Tatke et al., 2015 (*21*)
icd	qicd-F	GCGACCGGTGACAAAATCAC	Tatke et al., 2015 (*21*)
	qicd-R	GGGTTCTTCGGTACGCTCAA	Tatke et al., 2015 (*21*)
lpd3	qlpd3-F	CATGCGGCGGAGATGAAC	Tatke et al., 2015 (*21*)
	qlpd3-R	ACTTCCGGCTGGGTGTAGATG	Tatke et al., 2015 (*21*)
motA	qmotA-F	GTACCCCGGTGTGCTGAAGG	This study
	qmotA-R	AACAGGCCTTCCAGCTCGTG	This study
ostA	qostA-F	CCTGCCCTACAACCCAGGTG	This study
	qostA-R	ATGCTGCCGTCGTCATTGAA	This study
PA0794	qPA0794-F	CTGCCGGACAACATCACCAC	This study
	qPA0794-R	GGCCCATTTTCGCCAGGTAT	This study
PA4333	qPA4333-F	GCTCAACGGCAAGATGCTCA	This study
	qPA4333-R	AGCGTTTCGCCCTTGTTCAG	This study
pgi	qpgi-F	CCGACCACCTGCAACAACTG	This study
	qpgi-R	GGGATCAACTGGGTGCCTTG	This study
pilB	qpilB-F	ATCTCCGAACGACGCAAACC	This study

	qpilB-R	ACGATCTTCTCGCCCCACAG	This study
pilQ	qpilQ-F	AGCTATGCCCAGCCGATCAA	This study
	qpilQ-R	CAGGTCGATGACCACGTTGC	This study
pscP	qpscP-F	TGGCCAGTTGCAGATCGAGA	This study
	qpscP-R	GCTGTCCTGCTGCTGGTTGA	This study
rpoN	qrpoN-F	ATGACGAGTGGGACTTCACC	This study
	qrpoN-R	TCTTCGAGGTAGCCATCGTT	This study
rpsL	qRpsL-F	CGGCACTGCGTAAGGTATGC	This study
	qRpsL-R	CGTACTTCGAACGACCCTGCT	This study
sdhA	qsdhA-F	GCGCTGGAACTGCAAAACCT	This study
	qsdhA-R	CCGGGAAGTACAGGGTGTGG	This study
sdhB	qsdhB-F	GACCTCCTGCCCGTCCTTCT	This study
	qsdhB-R	GCGCTCTTCGGTCTTGGTGT	This study
sdhC	qsdhC-F	TTCCTCGGTATTGCCGTGCT	This study
	qsdhC-R	GACAGCAGGCCCCAGATCAC	This study
sdhD	qsdhD-F	CTCCGGCCTCTACGACTGGA	This study
	qsdhD-R	GCATCCAACCCTGCGAGAAC	This study
sucA	qsucA-F	CTGCAGCCAGCATCACATG	Tatke et al., 2015 (*21*)
	qsucA-R	CGAGATTGAGGCCCTTCTTG	Tatke et al., 2015 (*21*)
sucB	qsucB-F	ATCTCCAACGGTGGCGTGTT	This study
	qsucB-R	CATCATCGGCAGGATCACCA	This study
sucC	qsucC-F	AGCGAGTGGGTCGTCAAAGC	This study
	qsucC-R	TTGGCGTCGGTCTGGTAGGT	This study
sucD	qsucD-F	ATCCCGGGCTCCAACTTCAT	This study
	qsucD-R	GTAGGACACCACCGGCTTGG	This study
waaL	qwaaL-F	CTACGCCAGATCAGCGAGCA	This study
	qwaaL-R	CCTCCAGCGAAAAGCACACC	This study
wbpX	qwbpX-F	GAGACCATCCGCGACGAAGT	This study
	qwbpX-R	TCCTCCACCAGGTCCAGCTC	This study
wbpZ	qwbpZ-F	GCTCCGCCAGTACCGAGAAA	This study
	qwbpZ-R	ATCACCCCGACGAACAGGAA	This study
zwf	qzwf-F	GAAGGTATCTCCCTGCAAGT	McCaughey et al., 2013 (*46*)
	qzwf-R	GGTAGGTCTCGGAAAAACTC	McCaughey et al., 2013 (*46*)