Building a better biofilm - formation of *in vivo*-like biofilm structures by *Pseudomonas aeruginosa* in a porcine model of cystic fibrosis lung infection

SUPPLEMENTARY FIGURES AND TABLES

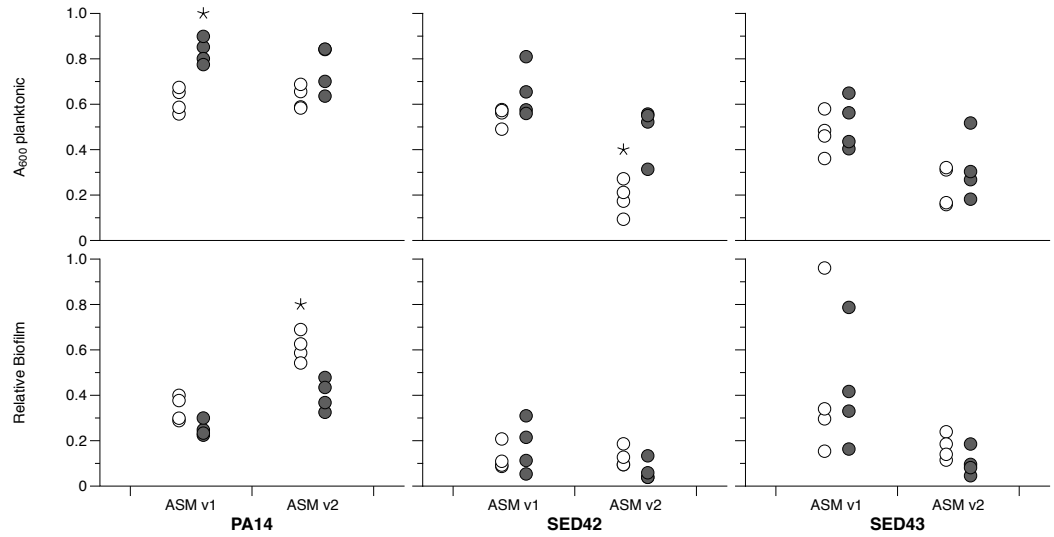

Figure S1. Growth of the *Pseudomonas aeruginosa* laboratory isolate PA14 and the Cystic Fibrosis (CF) isolates SED42 and SED43 in two versions of artificial sputum media (ASM) in 96-well plates with a peg lid to allow biofilm formation. Open circles: ASM with no glucose added. Closed circles: ASM supplemented with 3 µM glucose. Top panels show A_{600mn} of the planktonic subpopulations in each well after 24 h incubation at 37 °C. Bottom panels show relative allocation to biofilm on the plastic pegs, as measured by a crystal violet assay (A_{592nm} of crystal violet bound by each biofilm, divided by the A_{600nm} of the planktonic subpopulation of that well). Each data point is one replica population. ANOVA was used to analyse data and asterisks show conditions for each bacterial strain where growth or biofilm formation was significantly different when contrasted with that in ASM1 without glucose, using a post-hoc Tukey's test. For PA14, planktonic growth was enhanced by addition of glucose ($F_{1,12} = 23.7$, $p < 0.001$) but was not affected by ASM version (main effect $F_{1,12} = 0.73$, $p = 0.41$; interaction with glucose $F_{1,12} = 1.51$, $p = 0.24$). Biofilm allocation by PA14 was higher in ASM2 than ASM1 ($F_{1,12} = 56.0$, $p < 0.001$) and reduced by the presence of glucose (main effect $F_{1,12} = 28.7$, $p < 0.001$; interaction with ASM $F_{1,12} = 4.33$, $p = 0.059$). For SED42, planktonic growth was lower in ASM2 than ASM1 ($F_{1,12} = 33.0$, $p < 0.01$) and enhanced by the addition of glucose (main effect $F_{1,12} = 18.8$, $p < 0.01$; interaction with ASM $F_{1,12} = 4.69$, $p = 0.51$). Biofilm allocation by SED42 was unaffected by either ASM version ($F_{1,12} = 2.05$, $p = 0.18$) or glucose (main effect $F_{1,12} = 0.03$, $p = 0.86$; interaction with ASM $F_{1,12} = 2.06$, $p = 0.18$). For SED43, growth was lower in ASM2 than ASM1 ($F_{1,12} = 14.8$, $p = 0.002$) and unaffected by glucose (main effect $F_{1,12} = 1.17$, $p = 0.30$; interaction with ASM $F_{1,12} = 0.11$, $p = 0.74$).

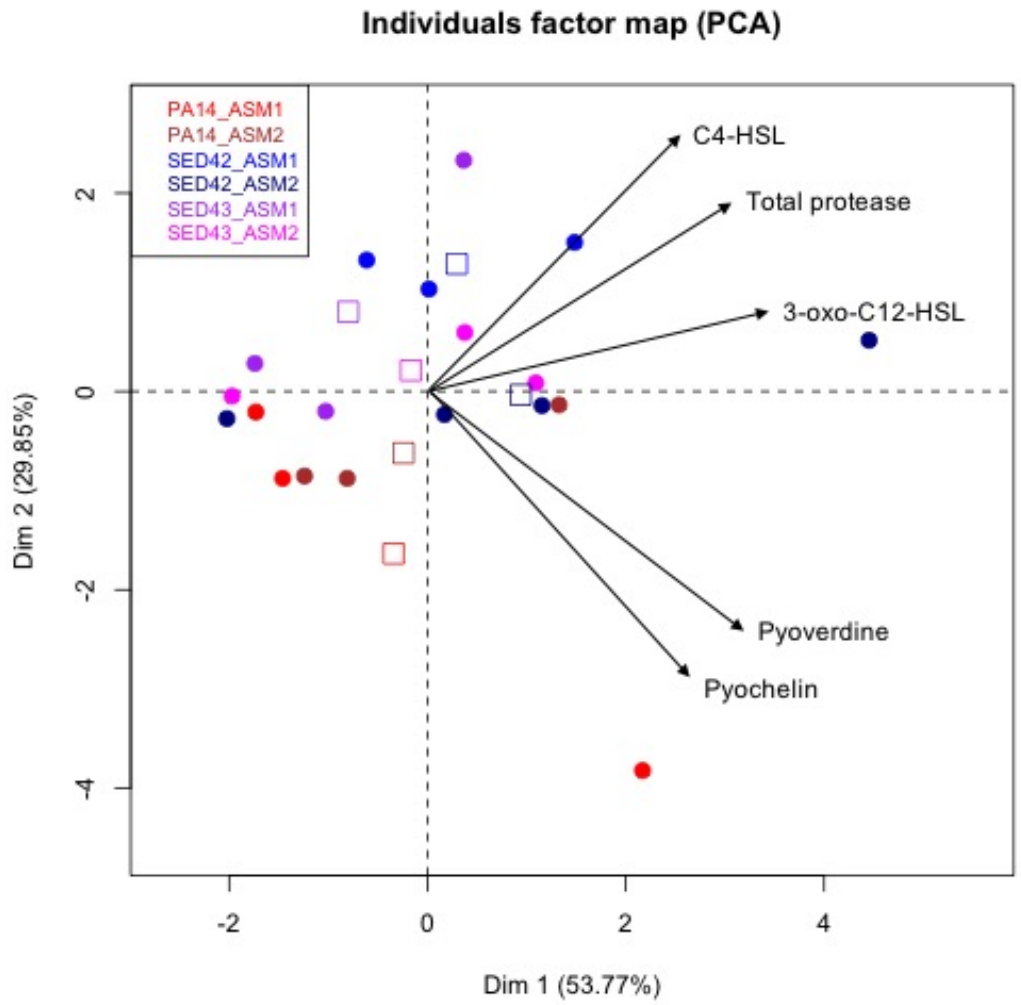

Figure S2. Principal component analysis of the virulence factor production data presented in Figure 1B for *Pseudomonas aeruginosa* strains. Only samples with data for all virulence factors were included. The circles show individual lung cultures, coloured to represent the *P. aeruginosa* strain cultured and the version of artificial sputum media (ASM) used. The squares are centroids. There is no clear clustering evident on the plot between ASM1 and ASM2 as the growth medium.

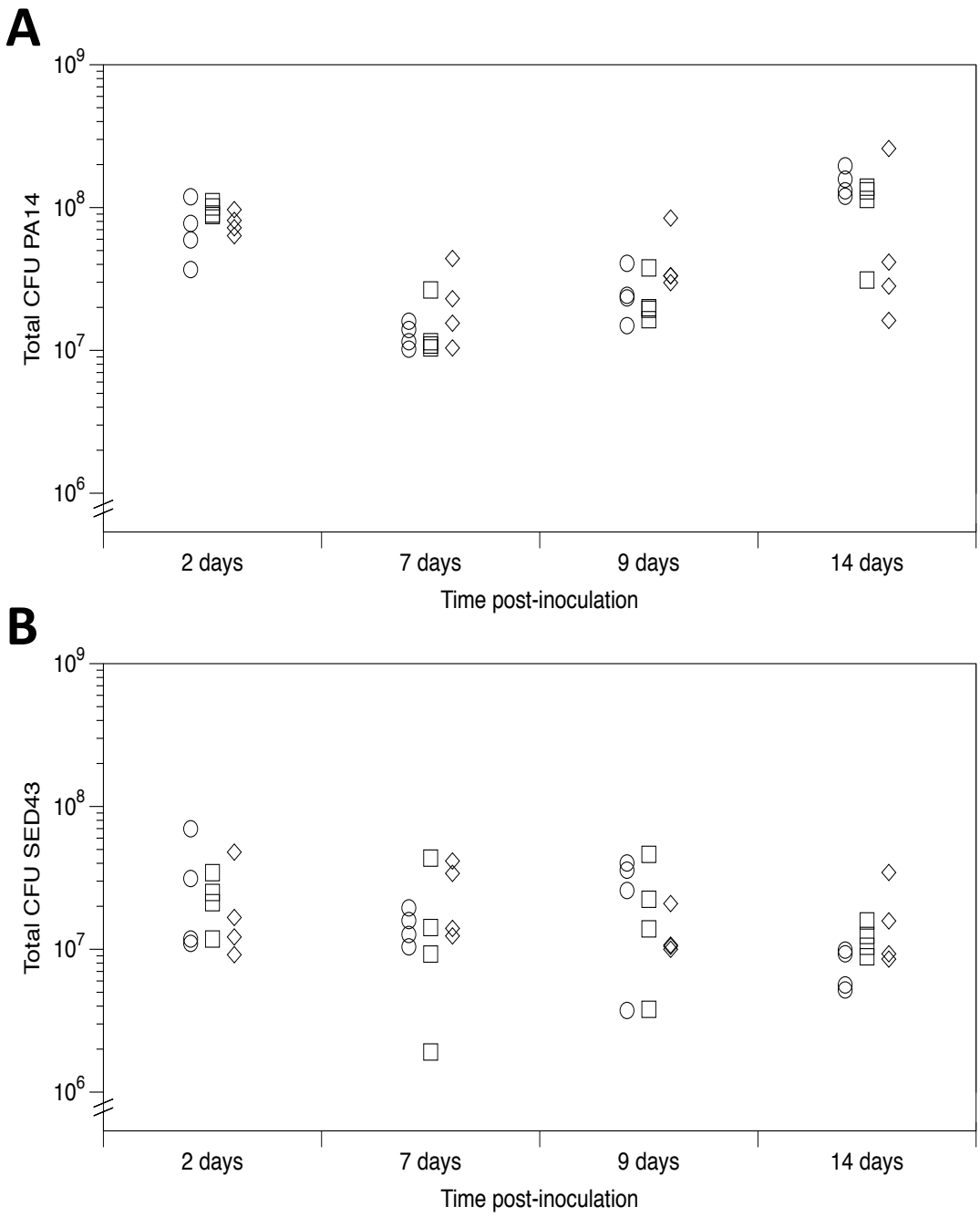

Figure S3. Growth of *Pseudomonas aeruginosa* PA14 and a representative chronic Cystic Fibrosis (CF) isolate: SED43, on pieces of *ex vivo* pig lung (EVPL) bronchiolar tissue plus artificial sputum media 1 (ASM1). Pieces of tissue from three independent lungs (different symbols) were inoculated with either PA14 or SED43 and destructively sampled at 2, 7, 9 or 14 d post infection (PI) at 37 °C to retrieve the biofilm. The graph shows colony-forming units (CFU) retrieved from individual EVPL biofilms at each time point. For PA14, after an approximate 1-log drop in CFU between 2 and 7 d PI, biofilm CFU increased again by 14 d, with some fluctuation between lungs. For SED43, CFU numbers were more consistent over time.

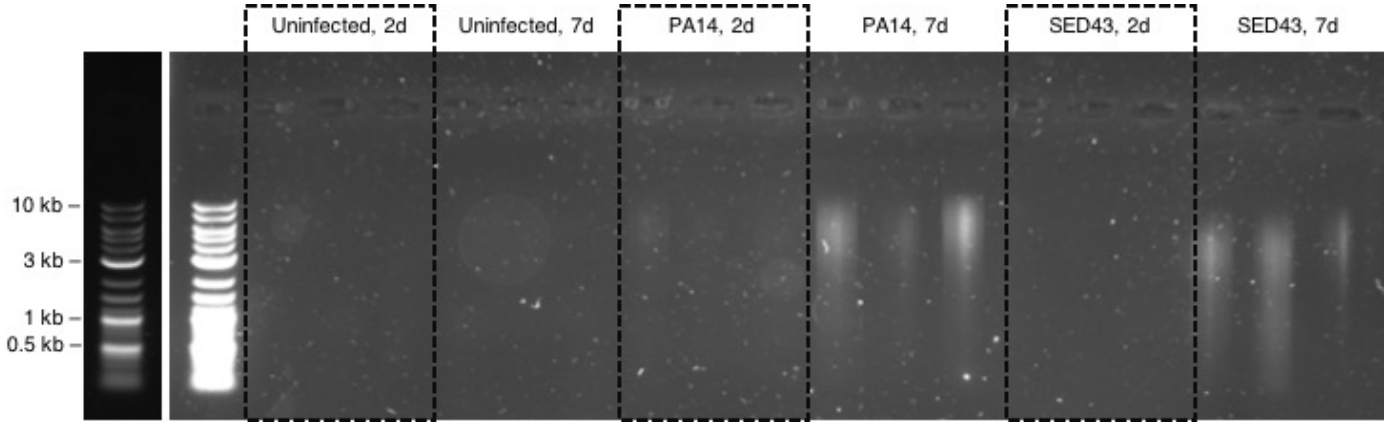

Figure S4. Detection of extracellular DNA (eDNA) in *Pseudomonas aeruginosa*-infected *ex vivo* pig lung (EVPL) + artificial sputum media 1 (ASM1). After 2 or 7 d growth, homogenate from EVPL cultures of PA14 and the clinical Cystic Fibrosis (CF) isolate SED43 (n=3 in each case) was centrifuged and the pellet run through a Promega Wizard Genomic DNA extraction kit. Recovered DNA was eluted in 50 µl water and 10 µl of the eluate run on an agarose gel. Uninoculated EVPL which had been incubated in ASM1 was also run through the extraction kit (n=3). No DNA was visible from uninoculated EVPL or EVPL after 2 d infection with *P. aeruginosa*, but after 7 d sufficient eDNA was present in *P. aeruginosa* biofilm to be visible on the gel. As the signal was so weak, the lane corresponding to the DNA ladder is shown in duplicate with different exposure times to facilitate contrast of the bands (NEB QuickLoad Purple 2-log ladder).

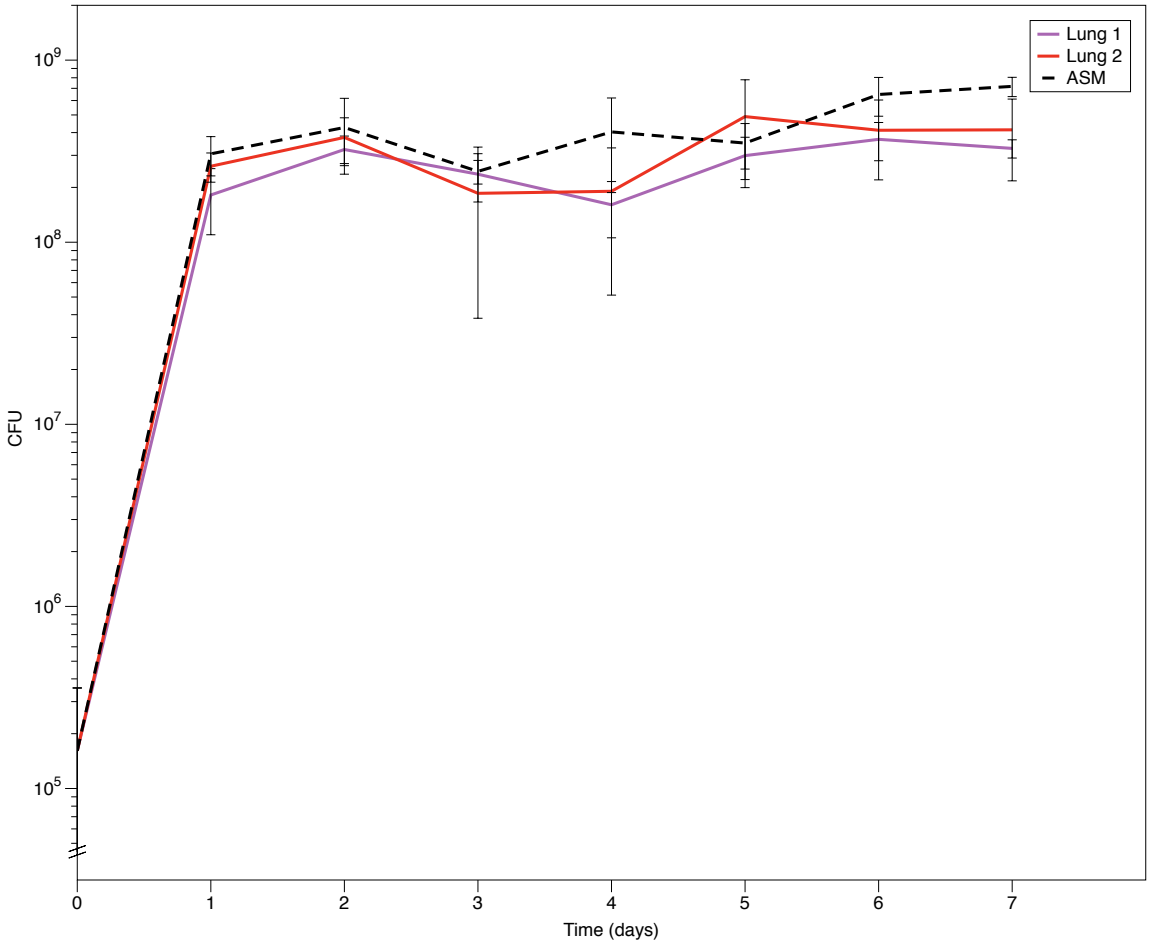

Figure S5. Growth of *Pseudomonas aeruginosa* laboratory isolate PA14 over 7 d in two independent lungs (red and purple lines) and *in vitro* artificial sputum media (ASM) (dashed black line). *P. aeruginosa* infected lungs and ASM controls were destructively sampled every day for 7 d to determine colony forming units (CFU) per lung and ml respectively. Three biological replicates were sampled per condition per day, and the graph shows the mean ± standard deviation.

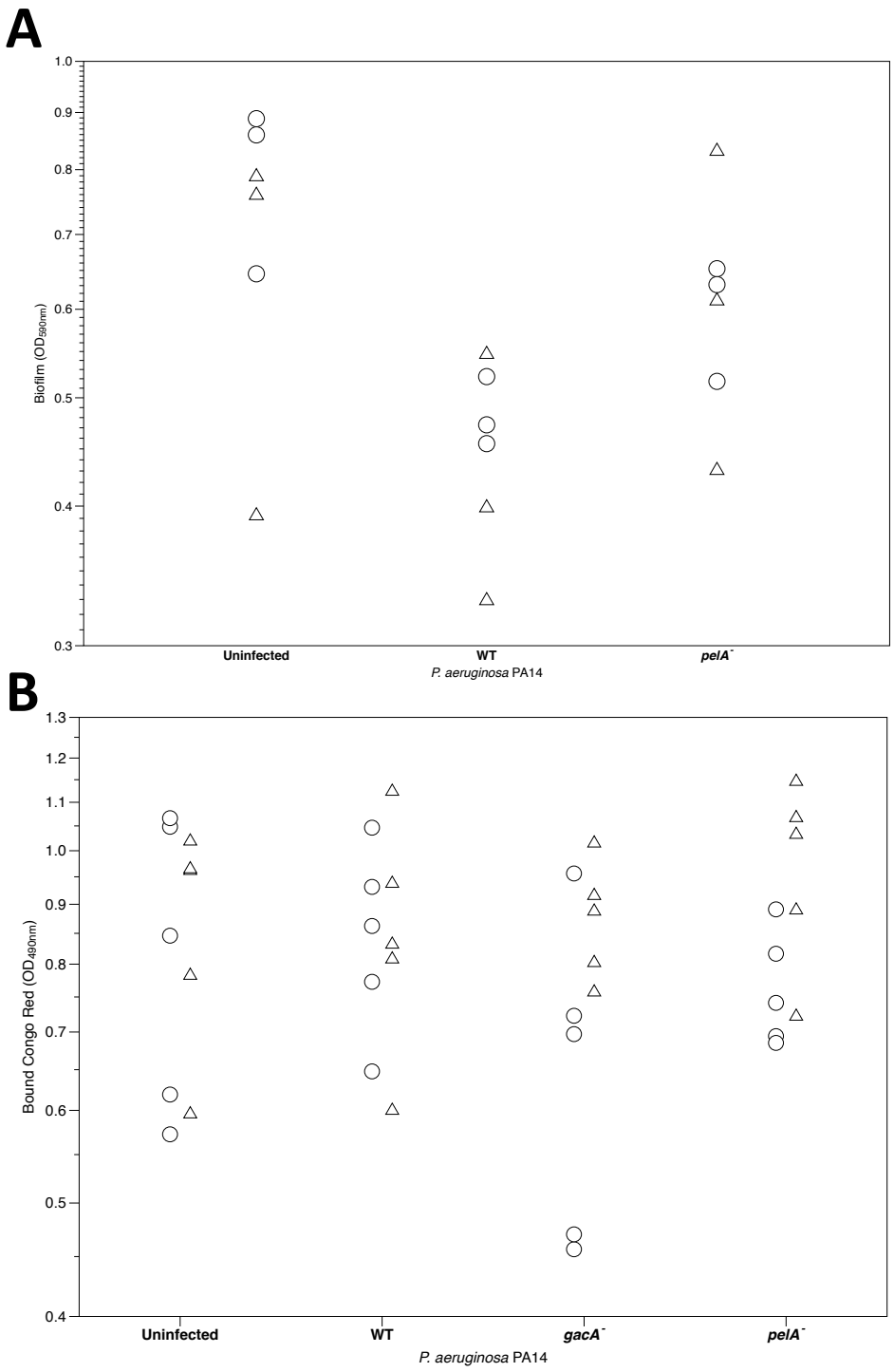

Figure S6. Measurements of characteristics of *Pseudomonas aeruginosa* biofilm on *ex vivo* pig lung (EVPL) bronchiolar tissue with artificial sputum media 1 (ASM1) formed by PA14 wild type (WT) and transposon insertion mutants. The EVPL tissue was infected for 2 d at 37 °C. Uninfected tissue was used as a negative control. (A) Biofilm measured from a crystal violet assay (A_{590nm}). Three replicate pieces from two independent lungs were infected with each strain. The highest absorbance measured was for the uninfected tissue sample, indicating that the crystal violet preferentially binds to the tissue over the biofilm. (B) Exopolysaccharide Pel production measured by a congo red assay (A_{490nm}). Five replicate pieces from two independent lungs were infected with each strain. There is no clear difference between the uninfected negative controls and infected tissue pieces, indicating the tissue also binds congo red. Both assays were considered unsuitable for EVPL biofilms.

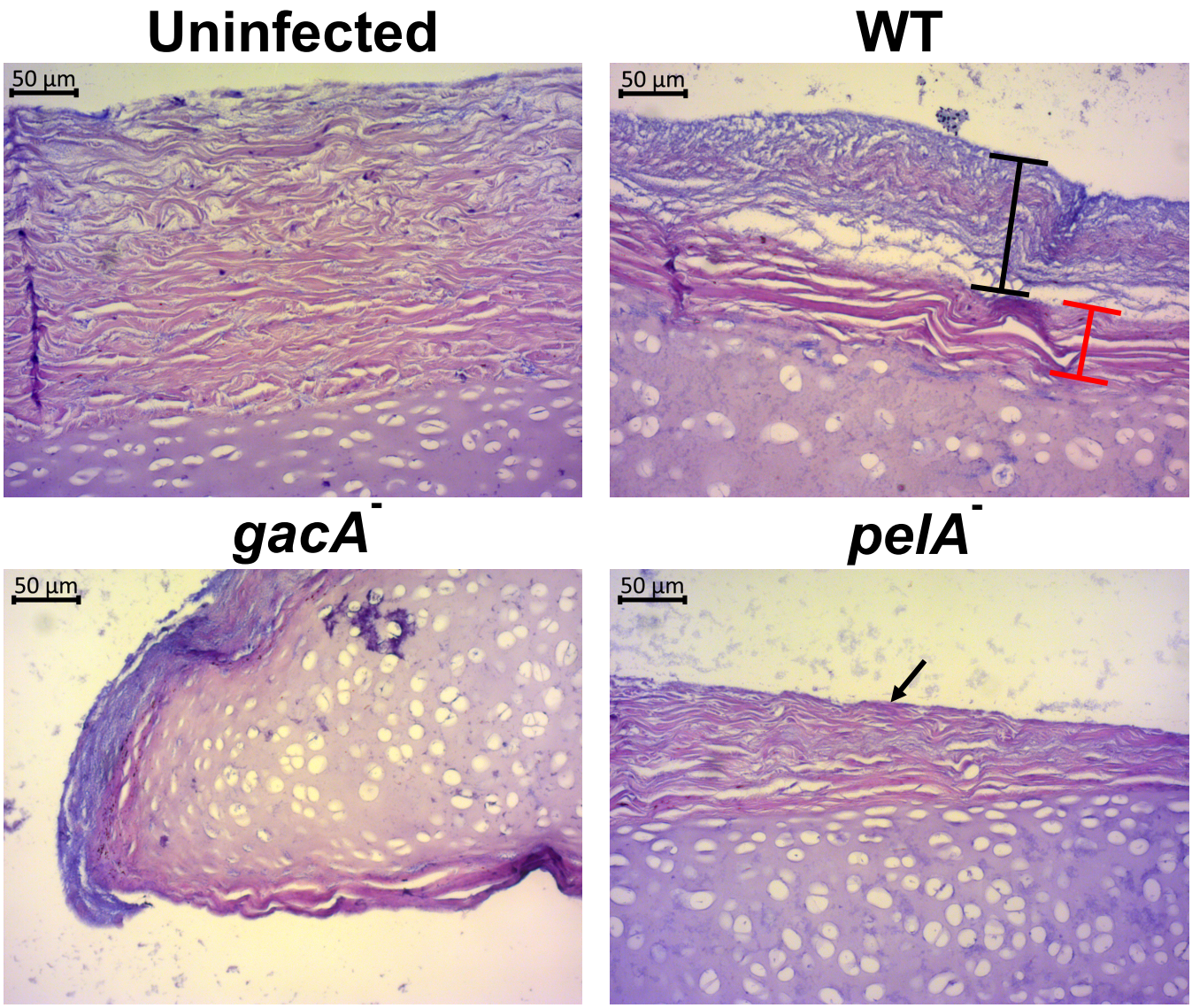

Figure S7. Hematoxylin & Eosin (H & E) stained sections of *ex vivo* pig lung (EVPL) bronchiolar tissue with artificial sputum media 1 (ASM1) at 7 d post infection (PI). Two independent lungs were infected for 7 d, fixed and H & E stained. Figure 4 images were taken from one lung and these images were taken from a second lung. The cartilage and tissue surface are stained pink and the bacterial biofilm purple. EVPL was infected with *Pseudomonas aeruginosa* PA14 WT and selected PA14 transposon mutants, with uninfected tissue as a negative control. All images are at x20 magnification. The presence of biofilm is shown by a black bar on the WT image, showing its depth, and the tissue shown by the red bar. The arrow shows the presence of the *pelA* mutant (as the mutant forms only a very thin layer on the tissue surface).

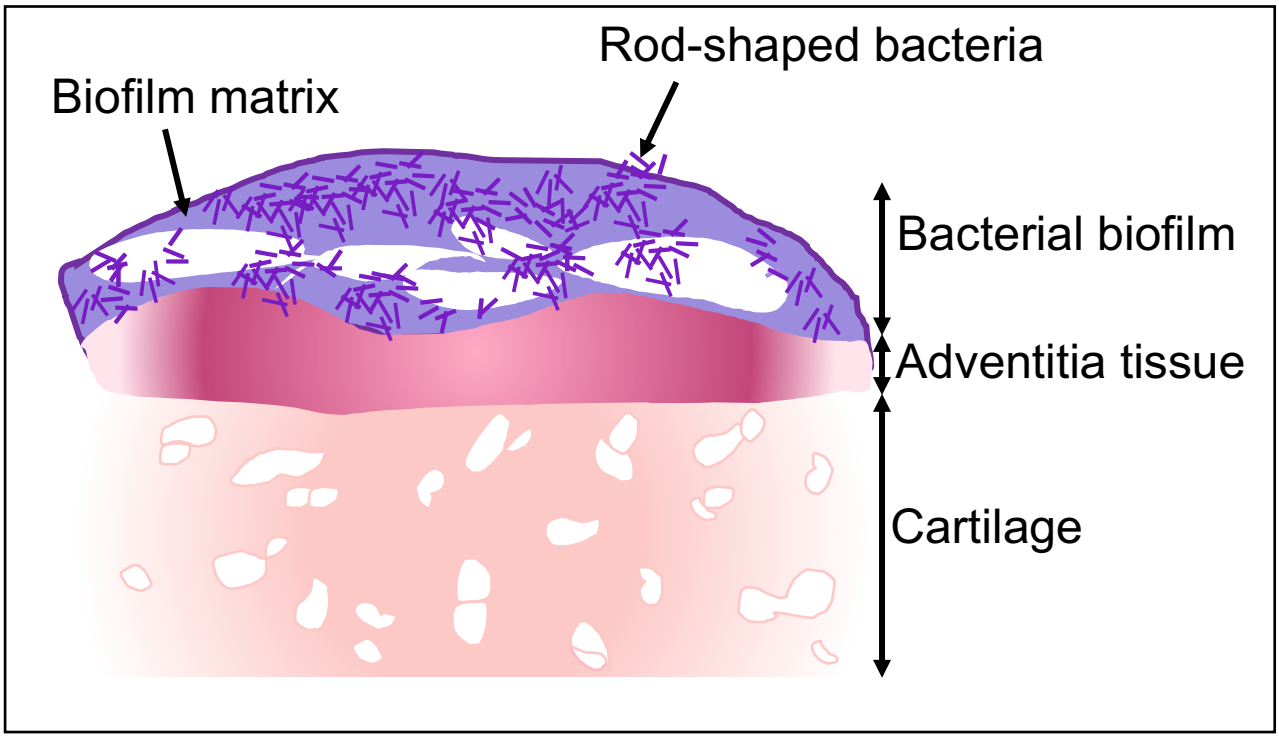

Figure S8. Diagram of *ex vivo* porcine lung (EVPL) bronchiolar tissue sections infected with *Pseudomonas aeruginosa* stained with Hematoxylin & Eosin (H & E). Tissue structures are labelled, stained pink, and the bacterial biofilm shown, stained purple.

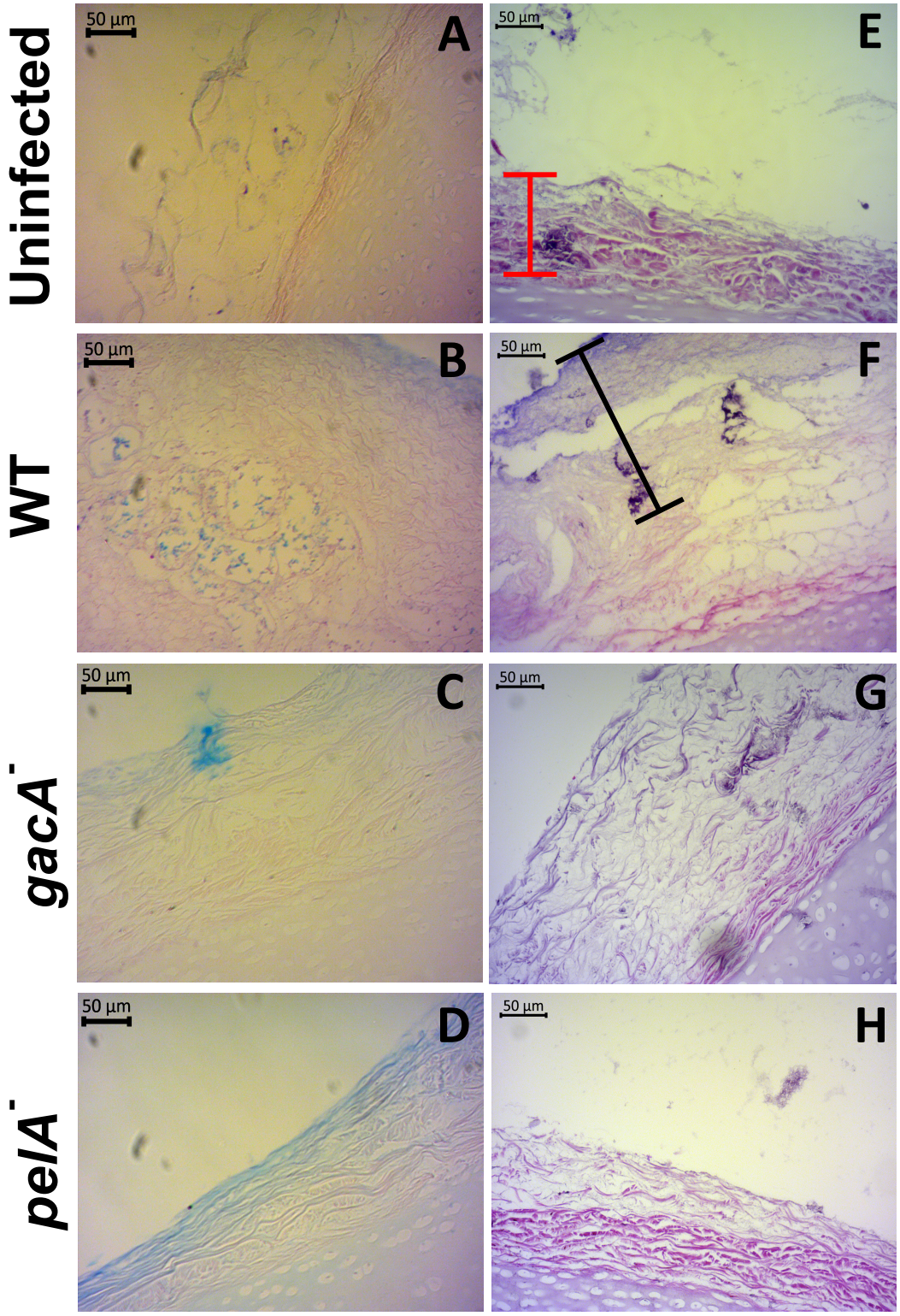

Figure S9. Representative micrographs of *ex vivo* pig lung (EVPL) bronchiolar tissue with artificial sputum media 1 (ASM1) 2 d post infection. EVPL was individually infected with wild type (WT) *P. aeruginosa* PA14 and selected transposon mutants for the PA14 strain. Uninfected tissue was used as a negative control. All tissue samples were paraffin embedded then stained with Alcian blue (A-D) or Hematoxylin and Eosin (H & E) (E-H). All images are at x20 magnification. The Alcian blue stains the biofilm matrix blue and the nuclear fast red counterstain stains the tissue red/pink. The H & E images shown the tissue surface is stained pink and the bacterial biofilm in purple. The black bar indicates the stained biofilm on the surface of the tissue and the red bar shows the tissue.

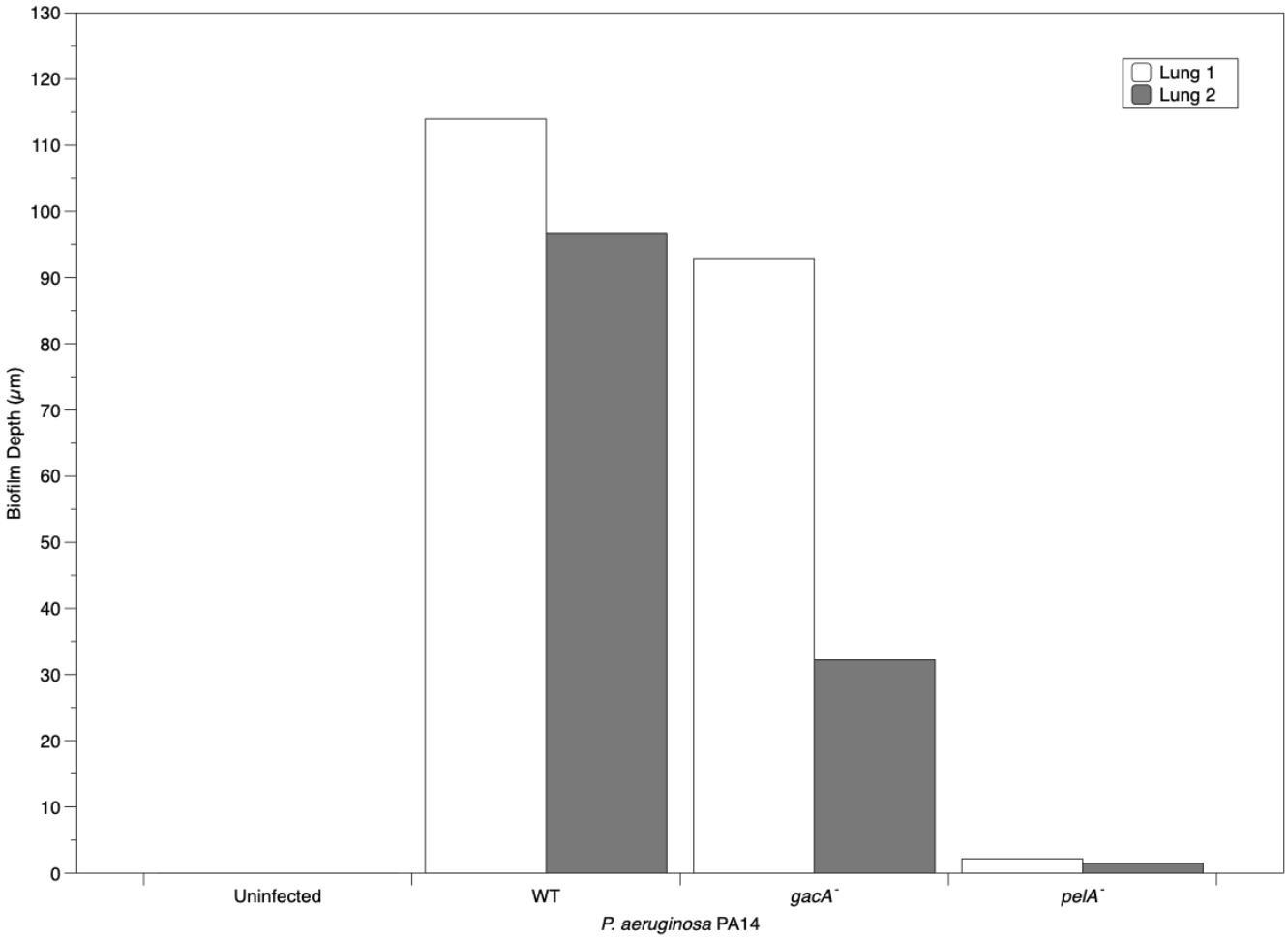

Figure S10. The depth (µm) of *Pseudomonas aeruginosa* biofilms formed on the surface of *ex vivo* pig lung (EVPL) bronchiolar tissue with artificial sputum media 1 (ASM1) 7 d post infection. Following infection of tissue pieces from two independent lungs with isogenic wild type (WT) *P. aeruginosa* PA14 and selected PA14 transposon mutants - uninfected tissue used as a negative control - tissue samples were paraffin embedded and Hematoxylin and Eosin (H & E) stained. Four measurements were taken along the biofilm from each H & E image using Zeiss Zen 2.3 pro software. The average biofilm depth per WT, mutant and uninfected tissue in the two lung repeats is shown.