An *ex vivo* cystic fibrosis model recapitulates key clinical aspects of chronic *Staphylococcus aureus* infection.

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Abstract

Staphylococcus aureus is the most prevalent organism isolated from the airways of people with cystic fibrosis (CF), predominantly early in life. Yet its role in the pathology of lung disease is poorly understood. In mice, and many experiments using cell lines, the bacterium invades cells or interstitium, and forms abscesses. This is at odds with the limited available clinical data: interstitial bacteria are rare in CF biopsies and abscesses are highly unusual. Bacteria instead appear to localise in mucus plugs in the lumens of bronchioles. We show that, in an established *ex vivo* model of CF infection comprising porcine bronchiolar tissue and synthetic mucus, *S. aureus* demonstrates clinically significant characteristics including colonisation of the airway lumen, with preferential localisation as multicellular aggregates in mucus, initiation of a small colony variant phenotype and increased antibiotic tolerance of tissue-associated aggregates. Tissue invasion and abscesses were not observed. Our results may inform ongoing debates relating to clinical responses to *S. aureus* in people with CF.

Impact Statement

Chronic bacterial infection is one of the main causes of declining lung function and morbidity in people with cystic fibrosis (CF). *Staphylococcus aureus* is the most prevalent organism isolated from airways of infants with CF. Yet its overall role in the pathogenesis of pulmonary disease is poorly understood. More accurate, high throughput models of CF lung infection are necessary to explore the interactions between *S. aureus* and host in this context. *In vitro* studies cannot accurately replicate chronic disease characteristics, such as presence of small colony variants, and animal models also do not represent features of clinical disease. Mice, in particular, are not

readily colonised by *S. aureus* and when infection is established it leads to the formation of abscess, a phenomenon not observed in the human CF lung. Here we show an existing *ex vivo* pig lung model of CF infection supports growth of *S. aureus* and that *S. aureus* demonstrates clinically significant characteristics including appearance of small colony variants, increased antibiotic tolerance and preferential growth as aggregates in mucus.

Data summary

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Introduction

Staphylococcus aureus (*S. aureus*) is currently the most prevalent pathogen isolated from the airways of people with cystic fibrosis (CF) (1). Historically, it is has been associated predominantly with paediatric pulmonary infection (2) yet most recent data shows that ~50% of even the oldest patients are now colonised (3). The presence of *S. aureus* in the respiratory tract varies considerably geographically and over time, and although prevalence appears to decrease with age (4) MRSA is most prevalent between 10-30 year olds(3). Determining the difference between colonisation and infection is both important and difficult. Nasal carriage of *S. aureus* among children with cystic fibrosis is common: Stone *et al.* (5) reported that 52.4% of the infants they studied harboured the organism. However, relatively high carriage rates in healthy children have also been recorded (6). Further, expectorate is difficult to collect from infants, so samples are usually collected by oropharyngeal swab, but presence of

organisms in the upper respiratory tract is not always indicative of lower airway infection (7, 8).

Moreover, the association of *S. aureus* with progressive lung disease – as measured by worsening lung function and the development of subsequent infection by the chronic CF pathogen *Pseudomonas aeruginosa* – is unclear (9). *S. aureus* is able to rapidly adapt to and persist in the CF lung environment (10), and worsening lung condition has been associated with the formation of *S. aureus* small colony variants (SCVs) (11). SCVs are known to remain in the lung longer than wild type (WT) bacterial cells (12) and demonstrate increased antimicrobial resistance (13). In studies of bronchoalveolar lavage (BAL) fluid from children aged 0-7 years, both Gangell *et al.* and Sagel *et al.* (14, 15) found that positive *S. aureus* culture was linked to a higher degree of airway inflammation, as measured by increased neutrophil count and IL-8. In another study, lung damage (bronchiectasis) in early CF was recorded by CT scan and *S. aureus* was the most commonly isolated organism (16). Conversely, in adult CF patients, *S. aureus* infections, in the absence of *P. aeruginosa,* are a marker of milder disease (1). There is also an indication that methicillin sensitive *S. aureus* may inhibit *P. aeruginosa*, thus delaying lung disease progression (17). The evidence that early *S. aureus* infection worsens prognosis for CF patients remains conflicting and warrants further study (3)(18).

The lack of understanding regarding the role of *S. aureus* in the development of lung disease in CF has led to debate over the use of anti-staphylococcal prophylaxis early in life. Studies by Ratjen *et al.* and Stutman *et al.* (19, 20) linked the use of broad-spectrum antibiotics to an increase in *P. aeruginosa* isolation. However, a recent

Cochrane review (21) showed no effect of anti-staphylococcal prophylaxis on *P. aeruginosa* colonisation at 3-4 years. There was a suggested trend towards higher rates of *P. aeruginosa* at 4-6 years but as the studies reviewed did not last more than six years, conclusions about the long-term effects of prophylaxis could not be drawn. A pragmatic randomized controlled trial is currently in progress (http://www.cfstart.org.uk).

In addition to the lack of clarity over the clinical consequences of *S. aureus* colonisation, there is also a gap in our mechanistic understanding of the microbiology of *S. aureus* in CF. The interaction of *S. aureus* with the pulmonary airway in CF, and its subsequent role in pathogenesis, is not clearly documented. CF epithelial cells have been shown to have an increased abundance of aGM1, a receptor that binds *S. aureus* and *P. aeruginosa*, compared with wild-type epithelia (22), and an *in vitro* study by Schwab *et al.* demonstrated that bacterial adherence to a bronchial epithelial cell line was significantly greater for CF *S. aureus* isolates than non-CF (23). McKenney *et al.* also show aggregates of *S. aureus* are visible on the surfaces of bronchial sections from children with CF (24). Yet, the only other study we found with direct evidence of *S. aureus* infection in human biopsy specimens, demonstrated that *S. aureus* did not adhere to the airway epithelium but was found aggregating within the mucus (25). Furthermore, these authors cited several studies reporting an ability of *S. aureus* to bind mucins (26, 27). It is obvious that better understanding of just how *S. aureus* colonizes the airway in CF is required, as are unambiguous data on the underlying mechanisms of pathogenesis and virulence, and the influence of *S. aureus* on subsequent infection by other microorganisms.

Mice are the most commonly used animal model of pulmonary infection in CF. Mouse models have been used to identify virulence-related genes in pathogens and to test novel therapeutic agents aimed at reducing inflammation or infection (28). However, mouse models present a number of challenges. In particular, mice do not develop spontaneous *P. aeruginosa* endobronchial infection, suppurative lung disease and mucus plugging of the airways, that are fundamental characteristics of human CF progression (29). *S. aureus* does not appear to readily colonize the airways of mice or produce an inflammatory response, even when clinical strains are used, in the presence of mucus (30). Furthermore, *S. aureus* forms severe abscess-like lesions in the mouse lung (31). When lung abscess does occur in humans, *S. aureus* is the most commonly isolated organism (32). However, lung abscess is rare in children (33) and even more so in people with CF: since the advent of neonatal screening and survival past infancy, abscesses are almost never observed in the CF population (34-36).

We previously developed a clinically relevant, high throughput model of chronic bacterial infection in CF. By combining sections of porcine bronchiole (obtained post-slaughter from a commercial abattoir) with culture medium which mimics CF sputum (artificial sputum medium, ASM (37)) we have shown that we can study another key CF pathogen, *P. aeruginosa,* in a physicochemical environment similar to that present *in vivo* (38-40). The use of CF-like growth medium combined with animal tissue with a greater structural, chemical and immunological similarity to human lung (41) means that our model is likely to facilitate a more human-like pathology of *S. aureus* than that observed in mouse models or *in vitro* studies. Using clinical reference strains and CF isolates of *S. aureus,* we can now demonstrate that in a CF-like environment, *S. aureus* shows non-invasive pathology. Bacteria grow as aggregates associated with the surface of bronchioles and in the

surrounding artificial sputum, without the formation of abscesses or other significant ultrastructural changes to tissue. In fact, we report a potential preference for aggregation in the surrounding mucus rather than growth associated with the bronchiolar surface. We also report the appearance of sub-populations of SCVs. Our work is consistent with *S. aureus* adopting a “persister” rather than “invader” strategy in CF. This stands in marked contrast to the pathology of this species in mouse and cell culture infection models, and underlines the importance of selecting models that reflect host-pathogen interactions more closely, in order to reliably study the role of specific bacteria in clinical disease progression. A better understanding of *S. aureus* pathology in the highly idiosyncratic environment of the CF lungs will inform optimised clinical responses to positive *S. aureus* cultures.

Materials and Methods

Bacterial strains. USA300 Los Angeles County clone (JE2, BEI resources) was used as an example of a well-documented clinical strain. 18 *S. aureus* stains isolated from 9 people with CF at Hospital Universitari i Politecnic La Fe were included as exemplars of CF associated strains. The strains used in this work were extracted for diagnostic purposes and sent to the microbiology service for routine analysis. Once analyzed, and instead of being discarded as usual, the strains were grown in tryptic soy broth and kept in glycerin. We chose isolates that represented a range of patient clinical presentation and bacterial phenotypes (e.g. weak or strong biofilm formation as measured by *in vitro* attachment assays, and isolates from patients during periods of stable presentation and episodes of acute exacerbation) (See Table S1).

Media and culture conditions for growth in the *ex vivo* pig lung (EVPL) model. For use in the lung model, bacterial stocks were grown overnight at 37 °C on lysogeny broth (LB) agar. Artificial sputum medium (ASM) was prepared according to Palmer *et al.* (37) with the modification that we removed glucose and supplemented with 20 μg ml^{-1} ampicillin. Our previous work suggested that glucose facilitated the growth of endogenous bacteria present in the lungs and that ampicillin helped to limit the growth of any resident bacteria in the lung that remained after sterilisation (38). We selected a concentration of ampicillin that provided the best possible coverage against endogenous populations but was sub-inhibitory for the *S. aureus* strains used (confirmed by standard MIC testing at the time of collection).

The EVPL model was adapted from our group's previous work (38, 39), which in turn built on prior use of pig lungs for non-CF studies (42). Briefly, lungs were collected from a local butcher (Steve Quigley and Sons, Cubbington, Warwickshire) as soon as possible following abattoir delivery, and processed immediately upon arrival at the laboratory. Previous antibiotic administration history of the pigs is not known, but use of antibiotics as growth promoters is banned in the EU, so use is restricted to prophylaxis and mass medication of herds only when infection is suspected. Approximately 5mm^2 sections of bronchiolar tissue were dissected from the lung under sterile conditions. During dissection the sections were washed three times with a 1:1 mix of RPMI 1640 and Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich), supplemented with 50 μg ml^{-1} ampicillin, and then rinsed once in sterile ASM without ampicillin supplementation. Bronchiolar samples were transferred to a clean petri dish and sterilized under a UV lamp for 5 minutes. Previous work has shown that tissue damage as a result of UV sterilisation is minimal (not visible using light microscopy)

198 (39). Samples were transferred to 24-well tissue culture plates: each well contained
199 400 µl ASM supplemented with 0.8 % w/v agarose to form a soft pad. Tissues were
200 inoculated with the appropriate strain of bacteria using a sterile hypodermic needle
201 (29G). The tip of the needle was lightly touched to the surface of the chosen *S. aureus*
202 colony, and then used to pierce the surface of bronchiolar tissue for inoculation. A
203 sterile needle was used for mock infection in uninfected controls. 500 µl of ASM + 20
204 µg ml^{-1} ampicillin was added to each well and the plate was sealed with a Breath-
205 Easier membrane (Diversified Biotech). Plates were incubated at 37 °C for up to 7
206 days and refreshed with 300 µl of ASM + 20 µg ml^{-1} ampicillin at 48 h. Following
207 incubation, tissue was rinsed in 1 ml phosphate-buffered saline (PBS) to remove
208 loosely adhering cells and processed for colony counts and microscopy.
209
210 **Bacterial load assay.** Tissue samples used to assay total bacterial numbers in tissue-
211 associated biofilm were homogenised individually in 1ml PBS in reinforced metal bead
212 tubes (Fisherbrand) using a FASTPREP 24 5G homogenizer (MP Biomedicals) for 40
213 sec at 4.0m sec^{-1}. Homogenates were serially diluted and aliquots were plated on LB
214 and mannitol salt (MSA) agar (Oxoid, Thermo Scientific) to obtain single colonies.
215 MSA agar is selective for *Staphylococcus* and was used to determine colony numbers
216 of test strains and ensure that negative controls did not contain any *S. aureus* already
217 resident in the lung tissue prior to experimentation. Non-selective LB agar was used
218 to check for contamination or growth of endogenous populations. When bacterial load
219 in ASM surrounding the lung tissue was measured, 300 µl of ASM was taken from
220 each well at the same time as tissue processing and transferred to a sterile Eppendorf
221 tube. Samples were vortexed, serially diluted and plated in the same way as tissue
222 homogenate.

LB plates were incubated aerobically at 37 °C for 18-24 h and MSA plates were incubated in at 37 °C 5% CO_2 for 24 h and then for an additional 48 h at room temperature in ambient CO_2 to allow visualisation of slow growing colonies. Total numbers of *S. aureus* recovered from the complete sample were calculated from tissue and ASM colony numbers combined.

Microscopy. Replica 5mm^2 bronchiolar tissue samples, dissected and inoculated as above, were preserved for hematoxylin and eosin staining. Samples were placed in individual tissue processing cassettes (Simport) and fixed overnight in 10% neutral buffered formalin (NBF) at approximately 20 times w/v. Longitudinal, step sections (100μm) were prepared for histopathology by an external service (University of Manchester). If samples required storage before transport, they were placed in 70% ethanol and kept at 4°C for up to 7 days. Sections were transported in PBS with residual NBF. In addition, 10 µl samples were taken from the ASM surrounding lung tissue, diluted 1:10 in PBS and 5 µl drops prepared for gram stain.

Microscopy was conducted with an Axio scope.A1 light microscope (Carl Zeiss) with AxioCam ERc5S digital camera and images processed in Zen Pro 2.1 Blue edition.

Colony identification. Colonies that were able to grow on MSA plates and ferment mannitol (indicated by yellow pigmentation of colony and surrounding clear zone) were regarded as *S. aureus*. Colony morphology was recorded by digital photography. The identity of colonies that were weak fermenters or small in size was initially confirmed using hydrogen peroxide to test for the production of catalase and a staphylase assay (Oxoid). If SCVs were suspected, colonies were recovered by aerobic incubation

overnight on Columbia Blood Agar and morphology was compared to the originally inoculated parent strain. PCR of large and small colonies from MSA plates was used to verify taxonomic identity. First, we performed colony PCR using *Staphylococcus*-specific primers (43). Once genus was confirmed, amplification of the 16S-23S rRNA intergenic spacer region and Sanger sequencing was used to confirm that SCVs were *S. aureus* (primers 5'-TGCCAAGGCATCCACCG-3' and 5'-GGCTGGATCACCTCCTT-3').

Determination of antibiotic susceptibility by microdilution. The minimium inhibitory concentration (MIC) of Flucloxacillin against a pair of representative MSSA isolates (FQ128 and FQ142) and the standard MSSA control strain, ATCC29213, was determined according to the british society of antimicrobial chemotherapy guidelines for microdilution (44) before examining antibiotic tolerance in the EVPL model. All isolates were from sputum samples collected from people with CF during stable presentation. All three strains demonstrated an MIC of <0.0625mg/l and were sensitive to flucloxacillin.

Antibiotic tolerance in the EVPL model. Infected lung bronchioles incubated for 48 h as described above. At 48 h 'pre-dose' tissue samples were homogenized and assayed as above. Biological replicates (4 bronchiolar sections for each strain and condition) were challenged with antibiotic: tissue samples were washed in PBS then placed in a new 24 well plate on agar pads. 500µl ASM alone was added for controls and 500µl ASM + either 0.25mg/l or 5mg/l flucloxacillin for antibiotic tolerance test samples. Concentrations were calculated from the recorded broth MIC of < 0.0625mg/l for the strains tested and the clinical target pharmacokinetic (PK) parameters. The PK

parameter for beta lactam efficacy is T>MIC with a target of 50% of dosing interval at a free plasma concentration 4-5 x MIC (45). 5mg/l was included to account for potential protein binding, which is 95% for Flucloxacillin (46). Plates were incubated at 37 °C. At 4 and 24 H post dose, treated tissues were washed in PBS to prevent any antibiotic carryover, and all samples homogenised and plated as described above. The bacterial loads in non-antibiotic and antibiotic treated tissues were compared.

Statistical analysis All data were analysed using RStudio (Mac OS X 10.6+ version 1.0.153) (RStudio, https://www.rstudio.com) using linear models followed by ANOVA and post-hoc Tukey HSD tests where appropriate, or using Kruskall-Wallis tests, as specified in the text.

Results

Clinical strains of *Staphylococcus aureus* are able to colonise EPVL. Data taken from preliminary experiments (Fig 1) showed that bacterial load (CFU) recovered at 48 h was similar for a large range of *S. aureus* CF clinical isolates, and comparable to Los Angeles County clone of USA300, a methicillin resistant (MRSA) clinical isolate known to form biofilm (47). Table S1 shows details of CF isolates. Samples were taken from people presenting either with acute exacerbation or in a stable condition (defined as chronic isolates). Isolates were grouped randomly and tested on three separate occasions. In each case lung samples were dissected from a single lung. Differences in growth were distinguishable between strains (ANOVA. $F_{2,40} = 1.9$, $p = 0.02$) but post hoc analyses showed no significant difference between any CF isolate and USA300LAC (see supplementary data). The median values for *S. aureus* load

297 recovered at 48 h were 1.64×10^5, 3.64×10^5 and 6.1×10^5 CFU respectively for lungs 1-
298 3. Growth on Staphylococcal selective media (MSA) was only observed on one of the
299 uninfected controls (Fig 1, Panel A).
300
301 ***S. aureus* growth in association with bronchiolar tissue depends on strain and**
302 **lung but trends are consistent overtime.** To verify the reproducibility of *S. aureus*
303 numbers recovered from bronchiolar tissue and the ability to observe differences
304 between strains, triplicate samples of USA300LAC and two clinical strains were
305 inoculated into three further lungs taken from different pigs (Fig 2) and incubation was
306 extended to 7 days. Clinical isolate FQ151 was compared with FQ184, these strains
307 were chosen as an appropriate example pair as they were both isolated from patients
308 with similar demographics, both MRSAs that form biofilm, and had both demonstrated
309 comparable rates of recovery at 48 h from the same lung (Fig 1). However, FQ184
310 was isolated during an acute disease exacerbation and FQ151 during stable
311 presentation (representing chronic infection or colonisation) (Table S1). It was
312 therefore hypothesised, that by comparing these two strains, differences between
313 exacerbation-associated strains and those apparently persisting asymptomatically
314 may be observed, and, if so, could be further investigated with a larger selection of
315 paired isolates. Figure 2 shows that USA300 was again able to establish in the lung
316 and mean *S. aureus* load recovered at 48 h was 6.8×10^6 CFU. Mean yields for clinical
317 strains at 48 h were 3.8×10^6 and 5.5×10^5 CFU for FQ184 and FQ151, respectively. By
318 7 days no colonies were recovered from tissue taken from one of the three lungs for
319 either clinical strain, and counts for clinical strains in a second lung were below or
320 close to the lower limit of detection. Average CFU counts at day 7 were 4.2×10^3 for
321 USA300, 7.9×10^3 for FQ184 and 8.1×10^3 for FQ151. The data were analysed by

ANOVA to test for differences between strains, lungs and days, and interactions between day*lung and strain*lung. Total bacterial load was significantly different once again between strains ($F_{2,40}$ = 12.0, p < 0.001) and between lungs ($F_{2,40}$ = 6.03, p = 0.005). There was a main effect of day ($F_{1,40}$ = 155, p < 0.001) and the magnitude of the drop between 48 h and 7 days did not vary significantly between lungs (day*lung interaction $F_{2,40}$ = 9.5, p = 0.110) or between strains (strain*day interaction $F_{2,40}$ = 1.73, p = 0.190). Different lungs did, however, affect the growth of the strains differently (lung*strain interaction $F_{4,40}$ = 2.62, p = 0.049).

***S. aureus* is visible as aggregates at the airway-tissue interface but does not appear to invade tissue.** Monitoring bacterial load within the model by CFU count shows that the model can maintain a *S. aureus* population at 48 h. In addition, it was important to investigate whether the consistent decline in CFU counts by 7 days was due to the inability of the model to support *S. aureus* or whether adaptation of *S. aureus* within the model prevented sufficient monitoring, for example if cells were growing in strongly adherent biofilm that had not been washed off, or as SCV or persister cells that were difficult to recover on media. Therefore, samples were histologically examined to gain a better understanding of growth characteristics in the model. Samples of the inoculated lung tissue (dissected from bronchi for original model) were washed in PBS and prepared for microscopy, by longitudinal, step section (100μm), at 2 and 7 days post infection. Histopathological staining (Figure 3 and 4) showed bronchial mucosa, represented by mature cartilage. In infected samples there is clear evidence of polymorphs and organisms, including, predominantly gram positive bacilli. Polymorphs and organisms are largely absent from uninfected controls with the exception of a small number of rod shape organisms (fig 3b). Gram positive

bacilli aggregate primarily at the tissue airway interface of infected lungs and their absence from the uninfected lung, (Figs 3a, 3b and 4a, 4b) strongly suggests that they are the *S. aureus* deliberately inoculated into test samples. Lung tissue remained largely intact across all tested isolates, even at 7 days. There was some disruption of tissue integrity at the surface, illustrated by the presence of fibrinous material (highlighted with *). None of the samples show any evidence of abscess, which would present as a clearly-defined, bordered structure. *S. aureus* is present only at the tissue-airway interface and does not appear to demonstrate tight association with tissue in the form of a mature biofilm, although more specific staining for biofilm would be required to confirm this.

Evidence of other organisms in some samples, for example rod shaped cells in the tissue infected with FQ184 at 48 h (arrow, Fig 3f, 100x magnification), is likely to be endogenous populations present prior to dissection (healthy lungs are not sterile) or contaminants not removed during sterilisation; they are also present in the uninfected control (arrow, Fig 3b). Interestingly, when other bacteria are present, there appear to be distinct ecological niches within the lung environment with gram positive bacilli clustered primarily at or near the surface, and rods present embedded within smooth muscle and connective tissue. This supports much of the current understanding of the structural architecture of polymicrobial communities and it would be useful to study this further especially as the presence of other bacteria may affect the ability of *S. aureus* to survive and adapt within the model. By day 7, cells in clusters of FQ151 were small and poorly stained, and thus difficult to image (Fig 4d). This may indicate the cells are no longer viable and explain the lack of colonies recovered on plates (Fig 2). Although inconclusive, the images appear to suggest that there is still a presence

of bacterial cocci in association with the lung tissue at 7 days post inoculation, despite poor recovery of colonies on selective media. It highlights that sputum sampling and colony count alone cannot reliably determine the presence of *S. aureus* populations that may be viable but non-culturable. It would be useful to further investigate, perhaps with the use of Staphylococcus aureus specific fluorescent probes or live dead staining in order to confirm the identity and viability of the cells.

***S. aureus* aggregates in artificial sputum surrounding lung tissue.** Initial experiments (Figs 1, 2) only recorded bacterial burden recovered from washed tissue pieces. The histology suggests at least some *S. aureus* may be tightly associated with the tissue in the model and not removed during homongenisation. In addition, there is evidence in the literature to suggest *S. aureus* may preferentially bind to mucus plugs in the airways of the CF lung, rather than associating with the tissue surface (25). Both are possible explanations as to why, despite presumably being adapted to the lung environment, CFU loads associated specifically with the bronchiolar tissue showed such a marked drop by day 7.

A subset of clinical strains, two MRSA and two MSSA (methicillin sensitive) strains, were chosen to investigate growth in the surrounding ASM, and compared with USA300 (Fig 5). When bacteria were also recovered from the surrounding ASM and enumerated, the total CFU count from the sample (tissue plus surrounding ASM) was higher than the CFU count for tissue only (Fig 5a). Although ‘mucus plugs’ do not form in the model, aggregates of *S. aureus* cells were visible in Gram stains of surrounding ASM at day 7 for all strains tested (Fig S5) and by day 7, a greater proportion of the total bacterial population was consistently found in the surrounding ASM than in tissue-

associated biofilm (Fig 5b). The four biofilm forming strains all showed a significant increase in the proportion of recoverable cells in the ASM between 48 h and day 7 as measured by the Kruskal Wallis Test (FQ128 χ_1^2 = 7.44, FQ140 χ_1^2 = 11, FQ151 χ_1^2 = 7.1, USA300 χ_1^2 = 12.3; all *p*-values ≤ 0.008). FQ142 did not show a significant increase in ASM proportion between the two time points (Kruskal Wallis test, χ_1^2 = 1.2, *p* = 0.22). As FQ142 is not a biofilm former (Table S1), this is likely due to a higher proportion already in the ASM by 48 h. ASM is a minimal media and it is significant that there are viable cells are maintained in the ASM for 7 days with limited nutrient replenishment, suggesting the addition of lung tissue to the model is an important improvement.

Tissue associated populations demonstrate phenotypes consistent with chronic infection. The data collected by cell enumeration (Figs 1, 2 and 5), for growth and localisation of *S. aureus* in the model, suggest the existence of subpopulations present during chronic infection. These likely comprise aggregates of cells in the mucus and a smaller percentage of tissue or biofilm associated cells, which may be slow growing SCVs or non-growing persisters. If this is the case it would be consistent with hypotheses in the literature regarding *S. aureus* infection(48), and we would anticipate that there would be SCVs present in the model and the "persister" subpopulation would demonstrate increased antibiotic tolerance.

Small colonies (<50% diameter of usual *S. aureus* colonies) were observed on MSA plates after incubation for at least 48 h in 5% CO_2 at 37 °C for samples taken 7 days post inoculation. Example colonies were photographed (Fig S6) and some had weak catalase and coagulase results when tested, weak catalase is indicative of deficient heme production and associated with SCVs, as is weak coagulase. SCVs were

423 confirmed as *S. aureus* spp. using genus-specific primers (STaG) (43) followed by
424 sequencing of the 16s-23S intergenic region (Table S2).
425
426 **Antibiotic tolerance of tissue associated aggregates.**
427
428 Slow growing or biofilm bacterial populations may be tolerant to antibiotics and
429 implicated in the persistence of chronic infection. Tolerance of tissue associated
430 aggregates to flucloxacillin was assessed for a pair of clinically isolated MSSAs that
431 were susceptible to flucloxacillin in a standard MIC microdilution and had the same
432 MIC as the control strain ATCC29213 (<0.0625mg/l). They were isolated from people
433 who presented asymptomatically (without exacerbation) (Table S1). Lung tissue was
434 infected with *S. aureus* and incubated as described above. At 48 h, antibiotic test
435 samples were challenged with a single dose of either 0.25mg/l or 5mg/l (to account for
436 potential protein binding) flucloxacillin; control samples were maintained in ASM alone.
437 Tissue was processed at 4 and 24 h post dose and total *S. aureus* load recorded as
438 before (Fig 6a). As in previous experiments, uninfected control tissues showed no
439 growth on MSA plates. Clinically, bactericidal activity of an antibiotic is regarded as a
440 reduction of 99.9% (>3 $\log_{10}$ decrease) in the CFU/ml of the original sample. The data
441 presented in Fig 6b demonstrates that a mean decrease of >3 $\log_{10}$ was only achieved
442 against FQ142 when challenged with the higher concentration of 5mg/l flucloxacillin
443 for 24 h, despite all three strains demonstrating susceptibility in a standard MIC test.
444 For ATCC29213 there was no significant effect of dose (ANOVA, $f_{2,21}$=0.2 p=0.8) or
445 dosing interval (ANOVA, $f_{2,21}$=1.9 p=0.2) on CFU/ml. Although there was an overall
446 significant effect of dose on CFU/ml for FQ128 (ANOVA, $f_{2,21}$=5.8, p=0.01) and FQ142
447 (ANOVA, $f_{2,21}$=7.5, p= 0.004), post-hoc analysis showed this was not significant for

448 0.25mg/l (FQ128, p=0.8 and FQ142, p=0.2) and a mean decrease in CFU/ml of
449 >3log$_{10}$ was not achieved for either strain at this concentration (Fig 6b). Dosing interval
450 was found to be significant for FQ128 ($f_{2,21}$=7.2, p=0.004) but not for FQ142 ($f_{2,21}$
451 =0.03, p=0.97). The fact that the required bactericidal target was not reached for the
452 control strain, ATCC29213, and that the response of the two clinical isolates varied,
453 shows that standard MIC testing may not reliably predict clinical response to antibiotic
454 regimes. There was no significant decrease in bacterial recovery between pre-dose
455 and 24 h post dose when challenged with 0.25mg/l flucloxacillin and FQ142 showed
456 a potential to grow under these conditions, suggesting sub-inhibitory flucloxacillin
457 concentrations could lead to the development of resistance. Furthermore, although
458 5mg/l flucloxacillin had a significant effect on CFU/ml for both clinical strains, it failed
459 to cause a mean decrease in FQ128 CFU/ml of >3log$_{10}$, suggesting that some strains
460 could tolerate clinically relevant concentrations even when antibiotic protein binding is
461 accounted for. This was a very small sample set, and further investigation is
462 warranted, but, it indicates that maintaining plasma concentrations well above the
463 target of 4 x MIC maybe necessary for effective treatment of *S. aureus* infection in CF.
464
465 **Discussion**
466
467 **Growth and localization of *S. aureus* in EPVL.** There is an apparent contradiction
468 between the usual pathology of *S. aureus* in human CF samples (non-invasive) and in
469 standard laboratory models using cell culture or mouse lung infections. To address this
470 gap between the clinical norm and standard lab models, we grew a biobank of clinical
471 isolates in an *ex vivo* model of CF lung infection which carefully mimics human tissue
472 structure and the chemistry of CF lung secretions. *S. aureus* grew with reasonable

consistency, both associated with lung tissue, at the airway surface, and as bacterial aggregates in artificial sputum (ASM) around the tissue. *S. aureus* bioburden was maintained in EPVL over 7 days, although tissue-associated CFU numbers were diminished over this period and in general the bacteria showed a preference for growth as aggregates suspended in the ASM.

Defined biofilm was not investigated in histological samples, although there was evidence of potential biofilm architecture and increased structure in bacterial populations for some samples by 7 days compared to 48 h. Interestingly, we have recently reported that *Pseudomonas aeruginosa* does demonstrate extensive biofilm growth in the EPVL that can be observed using the same histological staining techniques (40). This is consistent with the available clinical evidence where extensive biofilms have not been described for *S. aureus* in the CF lung (49), but small, biofilm-like aggregates are reported in the sputum and on the surface of airways (24, 25, 50, 51). Consistent with growth as aggregates *in vivo*, there is evidence that *S. aureus* switches off the global regulator *agr* in CF (48).

We did not observe interstitial invasion by *S. aureus*. Generally, interstitial bacteria are rarely observed in biopsy samples from people with CF (even if significant interstitial inflammation is observed) and bacteria in the airways are confined in luminal mucus plugs or occasionally attached to defined foci of epithelial erosion; this lack of tissue invasion is consistent with the rarity of bacteremia in people with CF (24, 25, 52-57). CF lung disease thus presents as bronchiectasis with mucous plugging of small airways. In contrast, microscopy images presented in studies of murine pulmonary *S. aureus* infections typically show abscesses (a cavitating, pus-filled lesion within the tissue with a defined border: see Figure 2 in (31) and Figure 2 in (58), for examples) and lead to chronic

pneumonia (59). Clearly, there are significant differences in *S. aureus* pathology in mouse models *versus* the highly specialised environment of CF airways as approximated by the *ex vivo* pig lung model.

In light of this, it is significant that our results suggest preferential localisation of *S. aureus* in the ASM surrounding the tissue sections, as opposed to epithelial surface attachment, and we did not observe the appearance of abscess-like structures. *S. aureus* can cause lung abscess in humans – in fact it is one of the most common bacteria isolated from abscesses (60) – but abscesses are almost never observed in people with CF (56, 57). A specific Panton-Valentine Leukocidin positive strain of MRSA was associated with invasive, cavitating lung lesions in a CF centre in the USA in the mid 2000s (61), but this is unusual. Other than this specific outbreak, a literature search revealed only five cases of abscess among people with CF, only two of which were associated with *S. aureus*: one patient was co-colonized by *P. aeruginosa* and the other with *P. denitrificans* (34, 62). It should be noted that abscess formation is associated with the presence of neutrophils, our model uses post mortem tissue and, therefore, the effect of the host immune response is not observable. In addition tissue samples in our model are taken only from the bronchi wall and so may not capture abscess formation. However, given the rarity of clinical evidence for invasive *S. aureus* infection in people with CF, induction of abscess formation may not be clinically important in a CF model. Clinical observations are consistent with a study in which mucus hypersecretion was induced in cultured primary nasal epithelia cells to better mimic conditions in the CF lung: mucus presence led to *S. aureus* cells moving away from the epithelial cell surface and growing in the mucus (25) – as observed in our model.

Chronic infection phenotypes of *S. aureus* in EPVL. The tissue-associated subpopulation exhibited enhanced tolerance to flucloxacillin compared with standard susceptibility assay results. This is likely due to physiological cues from the lung environment and the appearance of a subpopulation of SCVs (11, 13, 63-65) but may also be influenced by the presence of biofilm structure matrix if it is present (which could usefully be explored in future work).

Small colony variants emerged once *S. aureus* isolates were passaged through the EPVL. The phenotypic development of SCVs has been linked to chronic CF infections (63, 64). The impact of SCVs on virulence and persistence is difficult to study *in vitro* as they rapidly revert to normal colony morphology when attempts are made to culture them *in vitro* (51) and usually requires the creation of artificially generated, stable SCVs which may not fully represent those generated by the *in vivo* environment(66). Notwithstanding these limitations, studies have linked the SCV phenotype with attenuated virulence (67) and a dampened host cytokine response (68, 69) – both changes consistent with adoption of persistence strategy. The ability of the *ex vivo* lung model to cue SCV emergence is likely to be a significant strength of the model, permitting a rapid means to generate this phenotype and allowing more extensive future research into the effects of SCVs. It will be important, however, to tease apart the relative contributions of the presence of tissue and the presence of sub-inhibitory levels of ampicillin on SCV emergence. Since completing this work, we have begun a more extensive investigation of methods to reduce the presence of endogenous bacteria from the lung tissue, and we have found that expedited access to lungs after slaughter allows us to obtain tissue which requires less rigorous control measures. Future experiments using EVPL with different or no antibiotic

supplementation will allow us to explore the impacts of tissue and antibiotic presence on microbial physiology.

Population heterogenicity of *S. aureus in vivo* and in EVPL. The evidence outlined above is consistent with *S. aureus* growing as phenotypically heterogeneous populations in CF – comprising tissue-associated, biofilm-like aggregates and mucus-embedded aggregates, with potential presence of SCVs. Persistence, antibiotic tolerance, gene expression and ability to be cultured *in vitro* are likely to differ between these subpopulations, and understanding the differences within heterogenous populations could improve understanding of virulence mechanisms. If this is the case, the *ex vivo* pig lung model will be valuable in future study for the generation of heterogenous populations that are hard to reproduce in animal and traditional *in vitro* models.

Strengths and limitations of the EVPL model. Our data reinforce the notion that host specific pathology is an important area of future study, with particular significance for *S. aureus*. Modelling *S. aureus* infection, in any tissue other than human biopsy, is likely to present differences and challenges. It is abundantly clear that both mouse and *in vitro* models fail to capture key features of pathology observed from human clinical data that are revealed by our EVPL model. Consequently, EVPL could fill an important gap in the toolkit for answering crucial questions about the microbiology and clinical impact of *S. aureus* in CF.

There are, of course, some areas where the model could be optimised for future use. Primarily, variability in *S. aureus* bacterial load was considerable. We inoculated tissue sections from single colonies, and not a standardised broth culture, as preliminary work

572 with *P. aeruginosa* in an earlier version of the model showed no significant variation in the
573 number of cells inoculated from single colonies, or the cell numbers recorded at 48 h (39).
574 Additionally, in CF there is unlikely to be a large burden of colonising bacteria (70). Given
575 the differences in the way that *P. aeruginosa* and *S. aureus* grow in the model, starting
576 inoculum may be a more significant determinant of the growth and survival of *S. aureus*
577 and standardising cell numbers could be important for future developments, for example
578 assessing the impact of antimicrobial agents on viable bacterial burden. More broadly,
579 better data on the variability in *S. aureus* aggregate size between foci of infection within
580 CF lungs, and between patients, would be useful for understanding *S. aureus* pathology.
581 In a study by Hirschhausen *et al.* (59), adaptive changes differed in patients infected with
582 the same *S. aureus* clonal lineage, indicating that individual host factors had an impact on
583 adaption. This is reminiscent of our results, which show significant variability between
584 lungs taken from different pigs (Fig 2).
585
586 We also stress, given the reported intraspecific phenotypic and genetic diversity of
587 bacteria within and between people with CF, and given observed differences in the growth
588 and localisation of a pair of isolates taken from stable infection and acute exacerbation
589 (Fig 2 and 3-4), future work could usefully explore a larger collection of isolates in more
590 depth.
591
592 **Conclusion.**
593 The *ex vivo* lung model is designed to maximise physicochemical similarity to chronically-
594 infected human CF airways. It is also high throughput and inexpensive, and because it
595 uses post-consumer waste from the meat industry, it presents no ethical concerns (38).
596 Our results from *S. aureus* infection of the *ex vivo* model reveal aspect of chronic CF

pathology which are not captured by existing “gold standard” animal models: aggregation of bacterial cells associated with tissue but also (perhaps predominantly) in mucus; the development of SCVs; and an increase in antibiotic tolerance. The potential preferential binding of *S. aureus* to mucus, and lack of tissue invasion, is a phenomenon that may have clinical relevance, especially given current questions about the role of *S. aureus* as a CF pathogen and the consequences of trying to remove it.

Author Contributions

FH & ES conceived the study. ES optimised EVPL for the present work with *S. aureus*, conducted experimental work, analysed data and drafted the manuscript. FH developed the EVPL model and contributed to manuscript preparation. NEH conducted experimental work (histological sample preparation and imaging), completed statistical data analysis and contributed to manuscript preparation. AHH and BCA conducted experimental work as part of student projects for the University of Warwick’s BSc(Hons) in Biomedical Science and MB ChB, respectively. MMH contributed to the development of EVPL, conducted experimental work and edited the manuscript. MATM contributed clinical isolates and associated data. ARS and MNH contributed clinical context for the study goals and interpretations, and contributed to manuscript preparation. All authors saw and approved the final manuscript draft.

Conflicts of Interest

The authors declare there are no conflicts of interest.

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Ethical approvals

All methods were carried out in accordance with institutional and national guidelines and regulations. Sample collection and protocols were approved by the Biomedical Research Committee of Research Institute la Fe (reference 2014/0563). Initial diagnostic plates of sputum samples were not stored and were discarded after strain isolation, and strains were anonymized: thus, according to the provisions of article 24 of Royal Decree 1716/2011 on biomedical research, it was not necessary to request the declaration of compliance with the organic law on data protection, nor informed consent.

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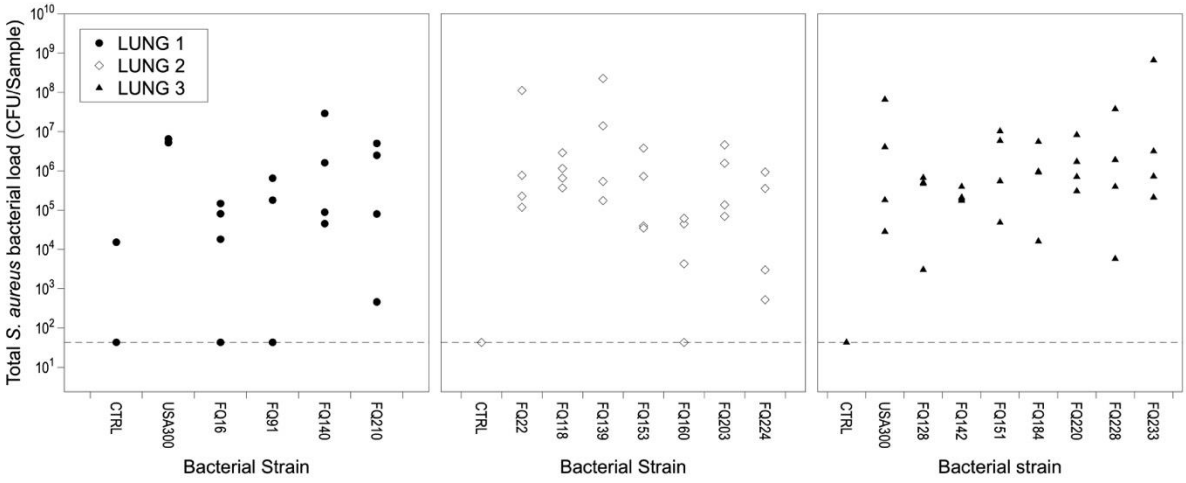
838 ## Figures and Tables



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841 **Figure 1. Recovery of *Staphylococcus aureus* following 48 H in the *ex vivo* pig lung model (EVPL).** Each *S. aureus* isolate was
842 inoculated into four replica bronchiolar sections of tissue (a single pair of lungs was used on each of 3 separate days).
843 Uninfected tissue was used as a control. Samples were destructively sampled at 48 h post infection. *S. aureus* bacterial load
844 was recovered on selective media (MSA) and is measured as total colony forming units per lung sample (Total CFU). Solid
845 lines indicate median values and the limit of detection is shown as a dashed line, no colonies were observed on plates where
846 data is at the limit of detection. USA300 is used as a representative control strain known to form biofilm. Clinical isolates
847 were supplied by the Instituto de Investigación Sanitaria La Fe (Table S1, supplementary material) and numbers are
848 designated as supplied. Analysis of untransformed data by ANOVA showed statistically significant differences in the bacterial
849 load attained by the different strains ($F_{7,32} = 1.90$, $p = 0.02$).

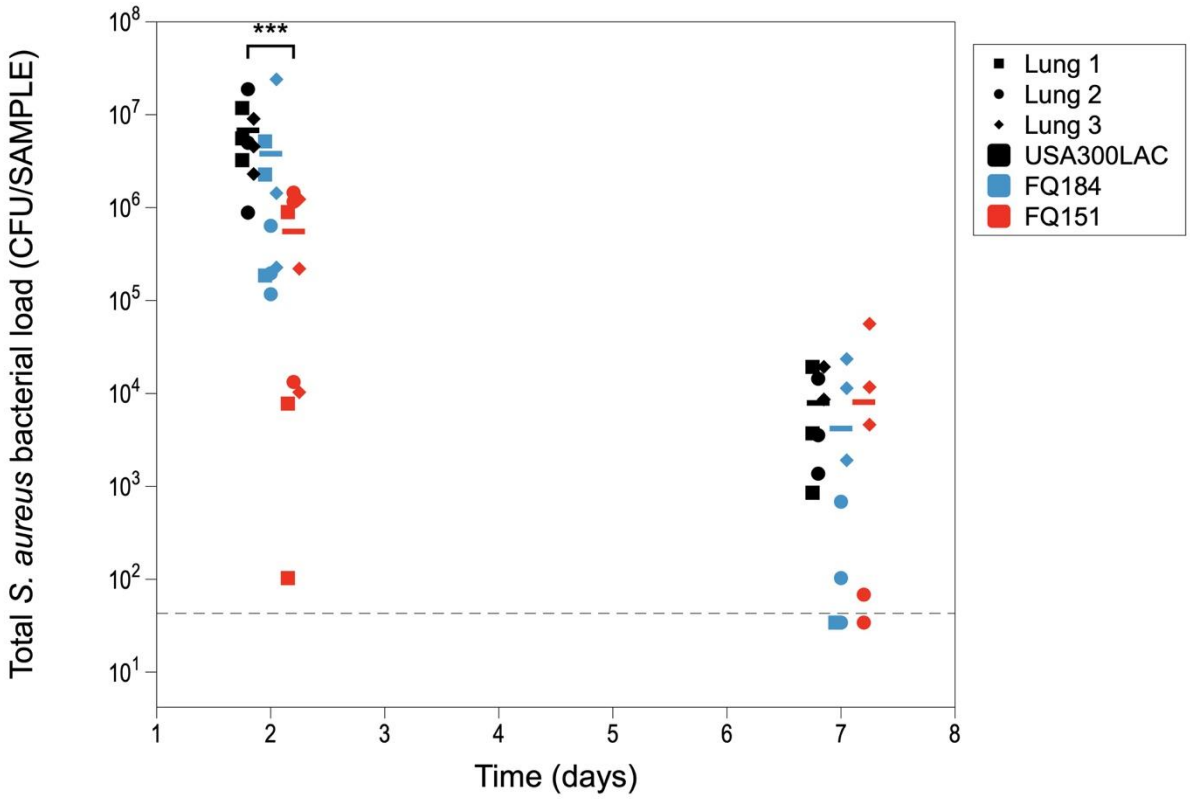


Figure 2. Recovery of an exacerbation vs chronic CF isolate of *Staphylococcus aureus* in the *ex vivo* pig lung model EVPL. *S. aureus* isolates were inoculated into six replica bronchiolar sections of tissue from each of three independent pairs of lungs, and samples were destructively sampled at 48 h and 7 days post infection by removing the tissue section from surrounding ASM and bead beating to release adherent bacteria. *S. aureus* bacterial load was recovered on selective media (MSA) and measured as total colony forming units per tissue sample (CFU). Triplicate lungs are indicated by point shape, and lines represent mean values. The limit of detection is shown as a dashed line. Colony counts on MSA, for uninfected tissue, were not recorded above the detectable limit at 48 H demonstrating that there was no inherent *S. aureus* presence in the lung tissue prior to inoculation. USA300 is used as a representative control strain. CF isolates supplied by the Instituto de Investigación Sanitaria La Fe and numbers are designated as supplied. ANOVA conducted on log-transformed data revealed significant main effects of strain ($F_{2,40} = 12.0$, $p < 0.001$), lung ($F_{2,40} = 6.03$, $p = 0.005$) and day, and a significant interaction between lung and strain ($F_{4,40} = 2.62$, $p = 0.049$). There was no significant interaction between day and lung ($F_{2,40} = 9.5$, $p = 0.110$) or day and strain ($F_{2,40} = 1.73$, $p = 0.190$).

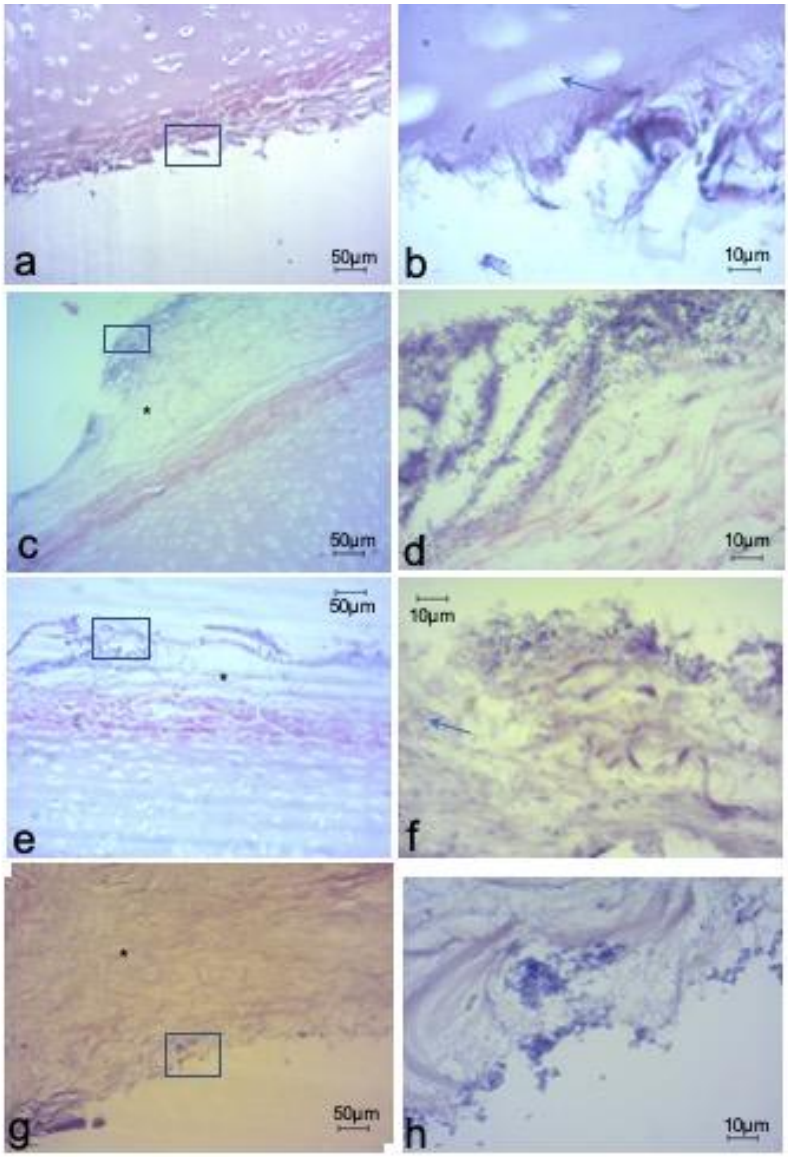


Figure 3. H & E stain of representative lungs at day 2.
Uninfected control (a,b) and 48 h post inoculation with FQ151 (c, d), FQ184 (e, f) or USA300 (g, h). x20 magnification (a, c, e and g). Highlighted area of interest (rectangle) is shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Cocci shaped cells are present in all inoculated tissue at x 100 (d, f and h) but not uninfected control (b). Disruption to bronchiole tissue is also evident in all infected samples (*), less extensive for FQ184 (e), a strain taken during acute exacerbation, but more marked for USA300 (g) and not seen in uninoculated control (a). Arrows indicate the presence of rod shaped bacterial cells in both uninfected (b) and inoculated (f) samples, suggesting the presence of endogenous lung species.

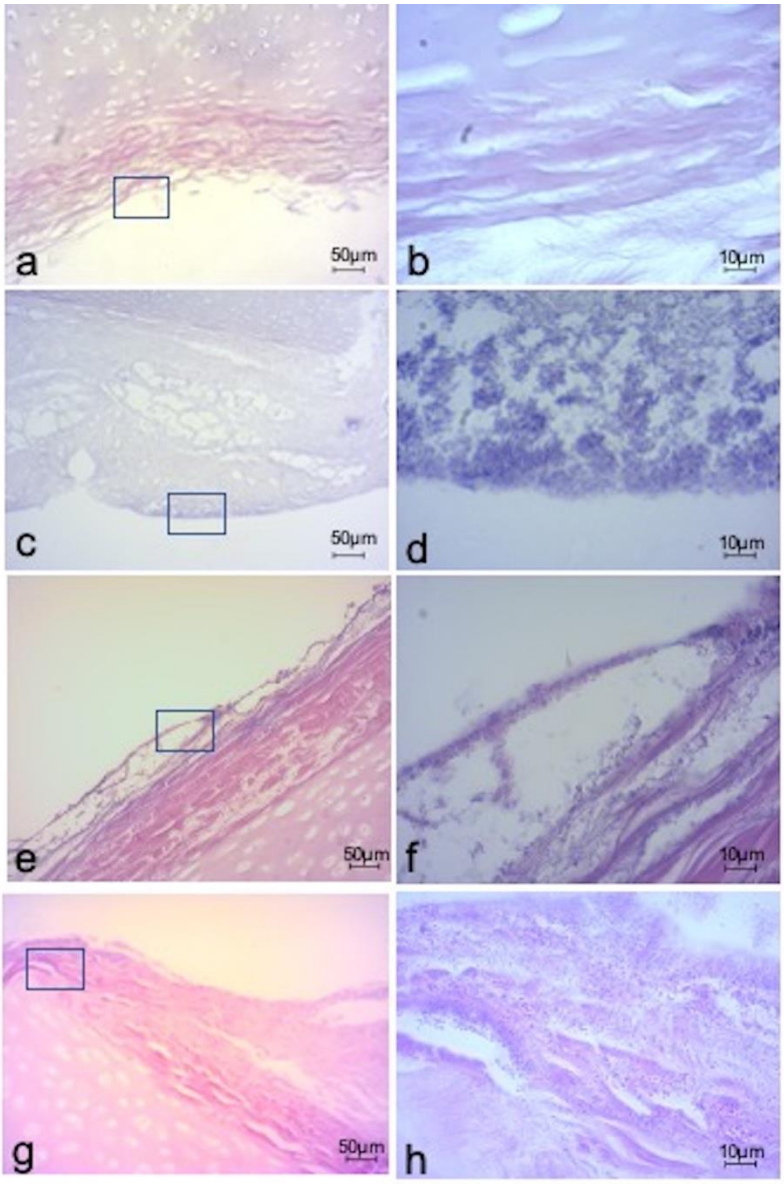
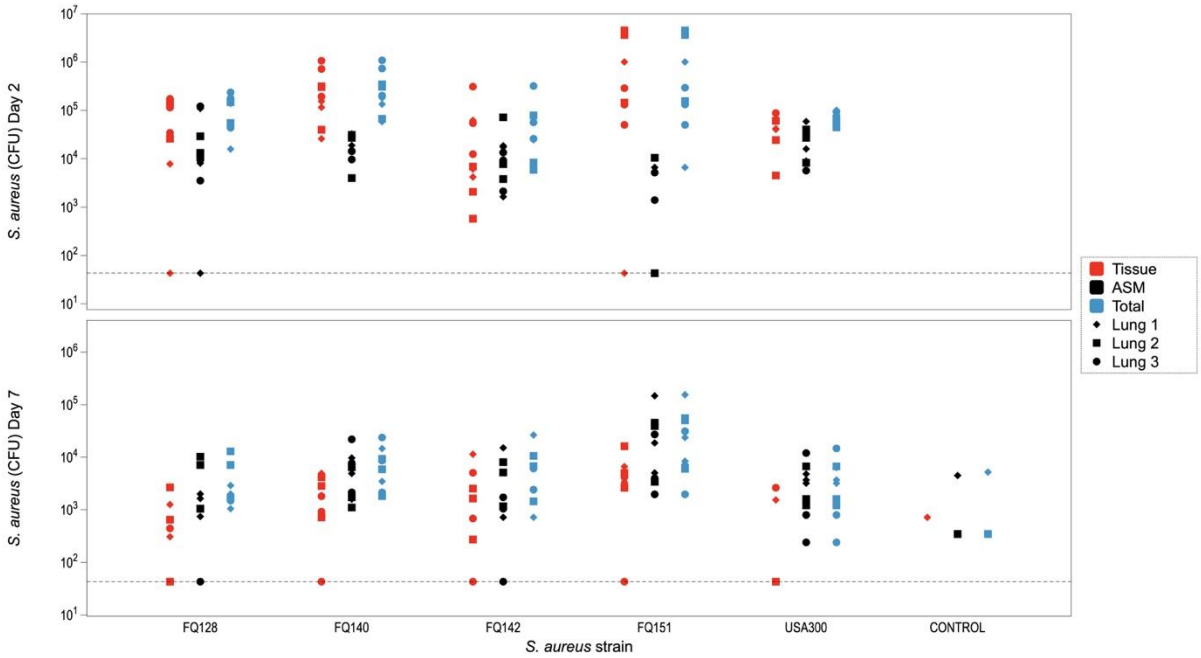


Figure 4. H & E stain of representative lungs at day 7.
Uninfected control (a,b) and 7 days post inoculation with FQ151 (c, d) FQ184 (e, f) and USA300 (g,h). x20 magnification (a,c,e and g). Highlighted area of interest (rectangle) is then shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Clusters of cocci shaped cells are present at or near tissue airway interface for all inoculated tissue at x 100 (d, f and h) but not uninfected control (b).

a)



b)

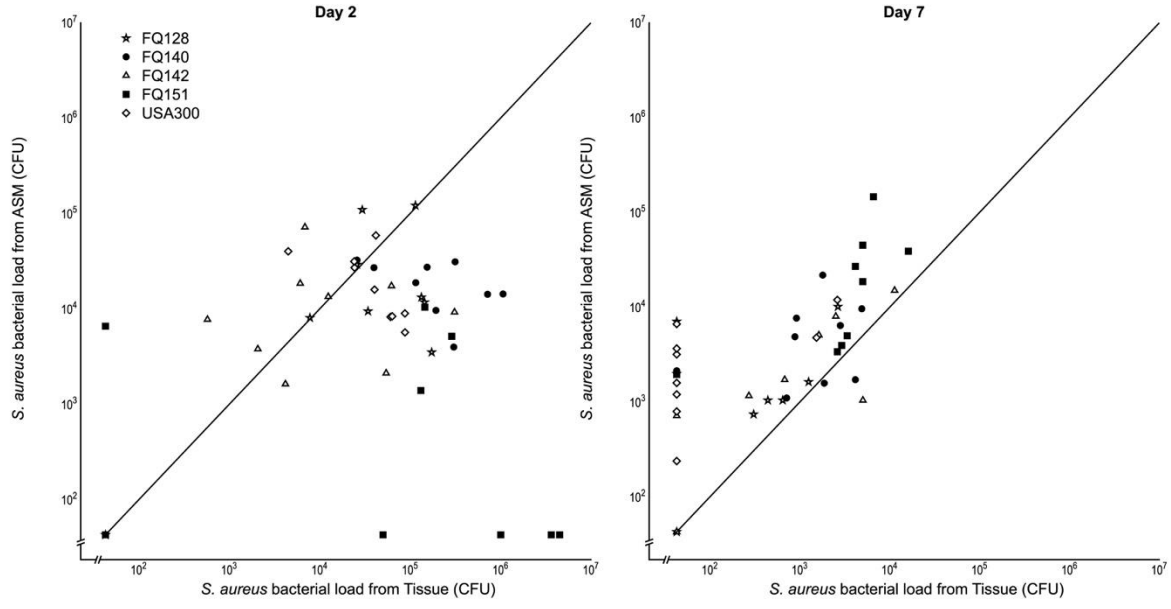
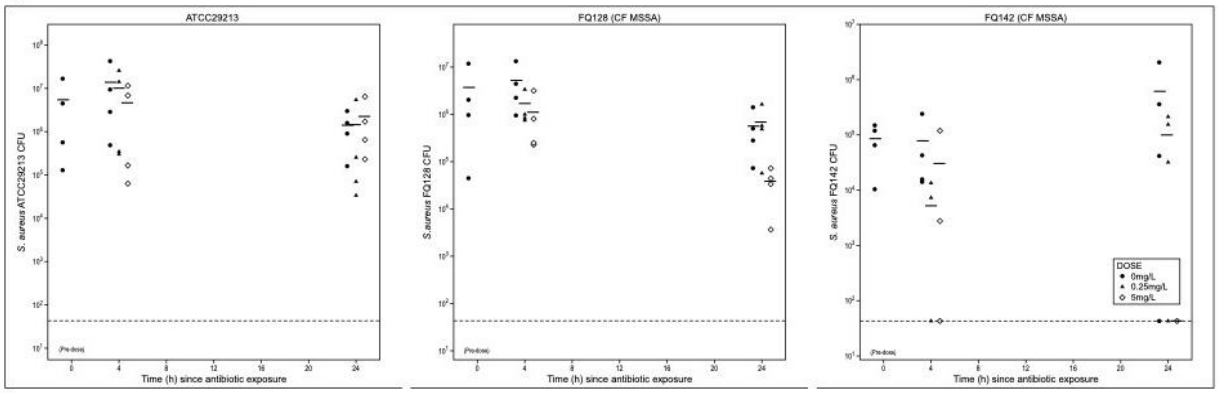


Figure 5. Location of *Staphylococcus aureus* in *ex vivo* pig lung (EVPL) model 48 h and 7 days: CFU and proportion of CFU recovered from Artificial Sputum Media (ASM) vs. tissue. *S. aureus* isolates were inoculated into six replica bronchiolar sections of tissue from each of three independent pairs of lungs, and samples were destructively sampled at 48 h and 7 days post infection. Aliquots of the ASM surrounding each sampled piece of tissue were also assessed for bacterial load. USA300 was used as representative control strain and an uninfected lung used as a negative control. Clinical isolates supplied by the Instituto de Investigación Sanitaria La Fe and numbers are designated as supplied. **(a)** *S. aureus* cell counts recovered on MSA agar from EPVL at 48 h and 7 days. Bacterial load measured as total colony forming units per tissue, surrounding ASM or total sample (CFU). Counts were taken from triplicate lungs, indicated by point shape. Non-parametric Kruskal Wallis tests

were used to test for differences in the proportion of bacteria in the surrounding ASM at 48 h and 7 days. Increases in proportional growth in ASM were found for strains FQ128 (Chi-squared = 7.44, p =0.006, df = 1), FQ140 (Chi-squared = 11, p < 0.001, df = 1), FQ151 (Chi-squared = 7.1, p =0.008, df = 1) and USA300 (Chi-squared = 12.3, p < 0.001, df = 1). FQ142 showed no significant increase in ASM proportion (Chi-squared = 1.2, p = 0.22, df =1). **(b)** Proportion of total CFU recovered from tissue vs surrounding ASM at 48 h and 7 days. Line shows equal CFU in ASM and tissue, for reference. Minimum limit of detection 4.3 x 10^1. Linear models on log-log transformed data showed a significant correlation between growth associated with tissue and growth in surrounding ASM at both time points. (Full details of ANOVA results in Supplementary information, R^2adj for models testing for effects of lung, strain, CFU lung and strain*CFU lung on CFU in ASM were 0.50 for 48 h data and 0.36 for day 7 data.

a



b

Time (h) since abx exposure	ATCC29213 survival (CFU) following flucloxacillin challenge (mg/l)		
	0mg/L	0.25mg/L	5mg/L
0 (pre-dose)	5.46E+06	NA	NA
4	1.38E+07	1.01E+07	4.64E+06
24	1.41E+06	1.45E+06	2.25E+06

Time (h) since abx exposure	FQ128 survival (CFU) following flucloxacillin challenge (mg/L)		
	0mg/L	0.25mg/L	5mg/L
0 (pre-dose)	3.71E+06	NA	NA
4	5.23E+06	1.70E+06	1.11E+06
24	5.65E+05	6.87E+05	3.84E+04

Time (h) since abx exposure	FQ142 survival (CFU) following flucloxacillin challenge (mg/L)		
	0mg/L	0.25mg/L	5mg/L
0 (pre-dose)	8.58E+04	NA	NA
4	7.81E+04	5.23E+03	3.04E+04
24	6.14E+05	9.97E+04	4.30E+01

Figure 6 recovery of *S. aureus* clinical isolates from EPVL following flucloxacillin challenge. Each *S. aureus* isolate was inoculated into replica bronchiolar sections of tissue from the same pair of lungs, and incubated as previously described for 48 h. Four replica samples were processed for counting of bacterial load, prior to antibiotic challenge (0 h, pre-dose). Remaining samples were moved to ASM containing either no antibiotic (●), 0.25mg/l (▲) or 5mg/l (◇)flucloxacillin and incubated for 4 or 24 h. Uninfected tissue pieces were used as control and processed as infected replica, no colonies were observed on recovery plates for uninfected controls. Lines represent mean bacterial load. All isolates were classified as sensitive by standard antibiotic susceptibility testing (MIC <0.0625mg/l). Bactericidal activity, (>3$\log_{10}$ in CFU) was only observed against FQ142 when challenged with 5mg/l flucloxacillin for 24 h (Fig 6b, highlighted). Antibiotic dose had no significant effect for ATCC29213 (ANOVA, $f_{2,21}$=0.2 p=0.8) for clinical strains there was an overall effect of dose: ANOVA, $f_{2,21}$=5.8, p=0.01 and $f_{2,21}$=7.5, p= 0.004 for FQ128 and FQ142 respectively. However post hoc analysis revealed there was no significance for either strain when exposed to 0.25mg/l flucloxacillin (FQ128, p=0.8 and FQ142, p=0.2).

Supplementary data.

Table S1. Clinical *Staphylococcus aureus* strains isolated from sputum samples from individuals with CF. Samples and health data collected and donated by the Instituto de Investigación Sanitaria La Fe, Valencia. Sample numbers are designated as supplied.

Sample	Patient Identifier	Patient age at sampling	Exacerbation	Biofilm	MRSA	Other microorganisms isolated
FQ16	51	21	No	Strong	No	*Pseudomonas aeruginosa, Aspergillus fumigatus*
FQ203	51	22	No	Moderate	No	*Pseudomonas aeruginosa*, *Burkholderia cepacia*
FQ22	21	17	No	Moderate	No	no
FQ91	80	7	No	Weak	No	no
FQ151	80	8	No	Moderate	Yes	no
FQ233	80	8	Mild	Moderate	Yes	no
FQ128	40	20	No	Strong	No	*Candida albicans*
FQ220	40	21	Yes	Moderate	No	*Candida albicans*
FQ140	99	8	No	Moderate	Yes	*Pseudomonas aeruginosa*, *Candida albicans*
FQ184	99	9	Severe	Moderate	Yes	*Pseudomonas aeruginosa*
FQ210	99	9	No	None	Yes	*Pseudomonas aeruginosa, Candida albicans, Haemophilus influenzae*
FQ142	25	13	No	None	No	*Candida albicans, Aspergillus fumigatus*
FQ228	25	14	Severe	None	No	*Candida albicans*
FQ118	70	14	No	Strong	No	no
FQ153	70	15	No	Strong	No	*Candida albicans*
FQ139	47	12	No	Moderate	No	*Aspergillus fumigatus*
FQ160	48	9	No	Weak	No	no
FQ224	48	10	No	Weak	No	no

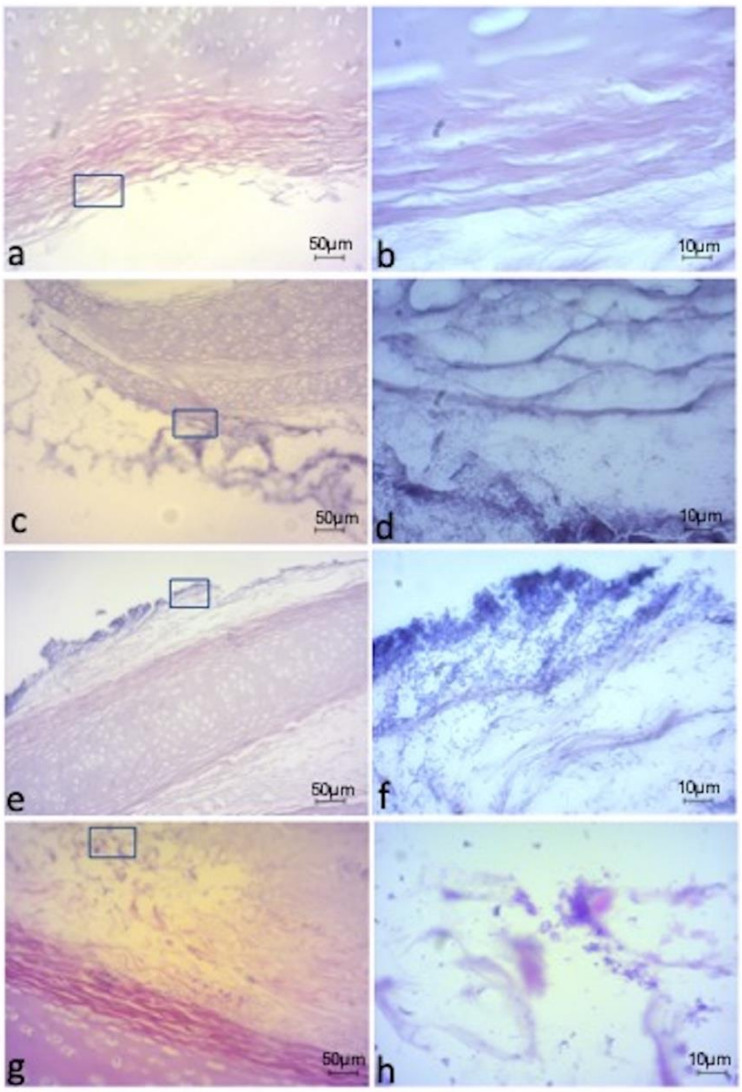


Figure S1. H & E stain of representative lungs at day 2.
Uninfected control (a,b) and 48 h post inoculation with FQ151 (c, d), FQ184 (e, f)or USA300 (g, h). x20 magnification (a, c, e and g). Highlighted area of interest (rectangle) is then shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Cocci shaped cells are present in all inoculated tissue at x 100 (d, f and h) but not uninfected control (b).

926

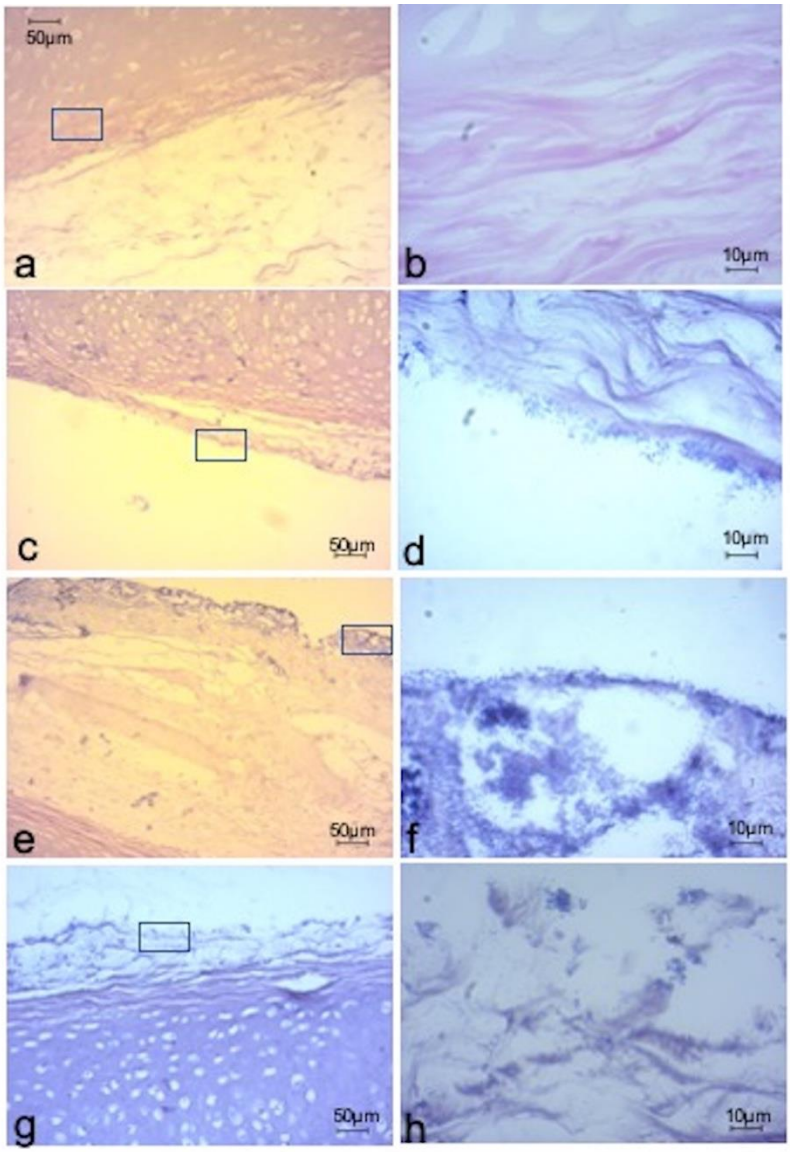


Figure S2. H & E stain of representative lungs at day 2.
Uninfected control (a,b) and 48 h post inoculation with FQ151 (c, d), FQ184 (e, f)or USA300 (g, h). x20 magnification (a, c, e and g). Highlighted area of interest (rectangle) is then shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Cocci shaped cells are present in all inoculated tissue at x 100 (d, f and h) but not uninfected control (b).

927

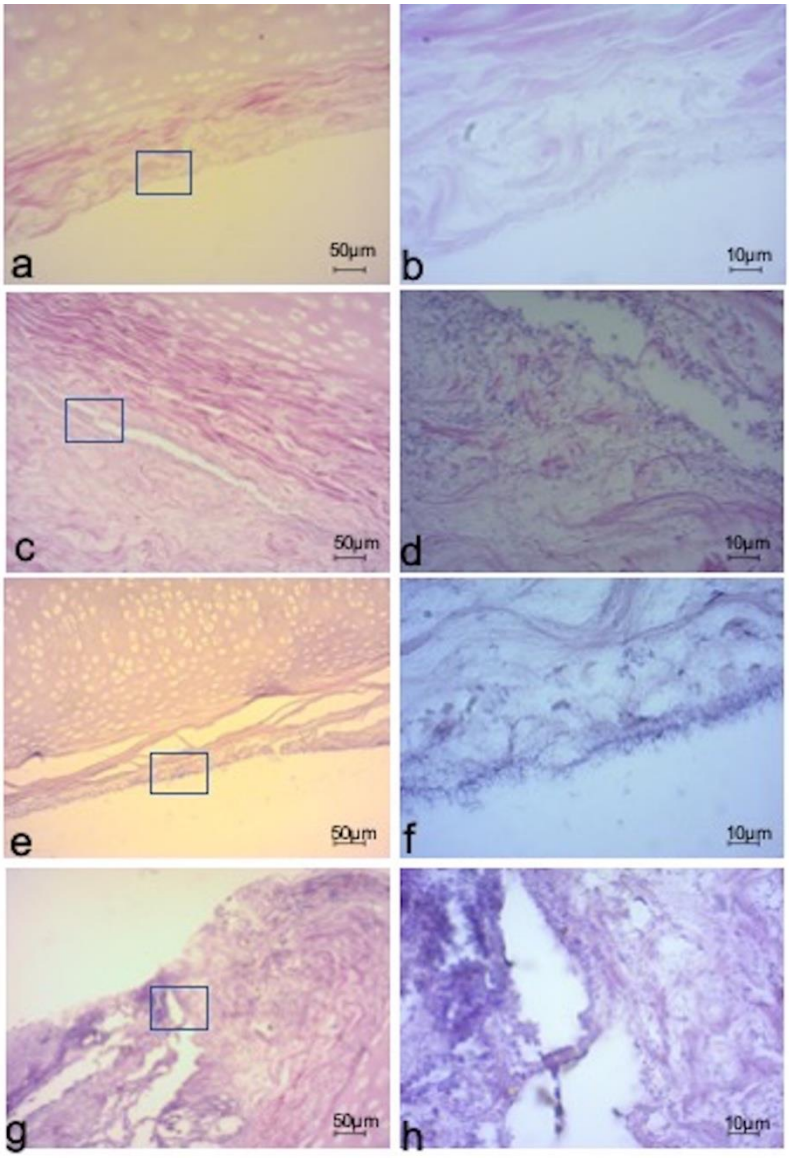


Figure S3. H & E stain of representative lungs at day 7.
Uninfected control (a,b) and 7 days post inoculation with FQ151 (c, d) FQ184 (e, f) and USA300 (g,h). x20 magnification (a,c,e and g). Highlighted area of interest (rectangle) is then shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Clusters of cocci shaped cells are present at or near tissue airway interface for all inoculated tissue at x 100 (d, f and h) but not uninfected control (b).

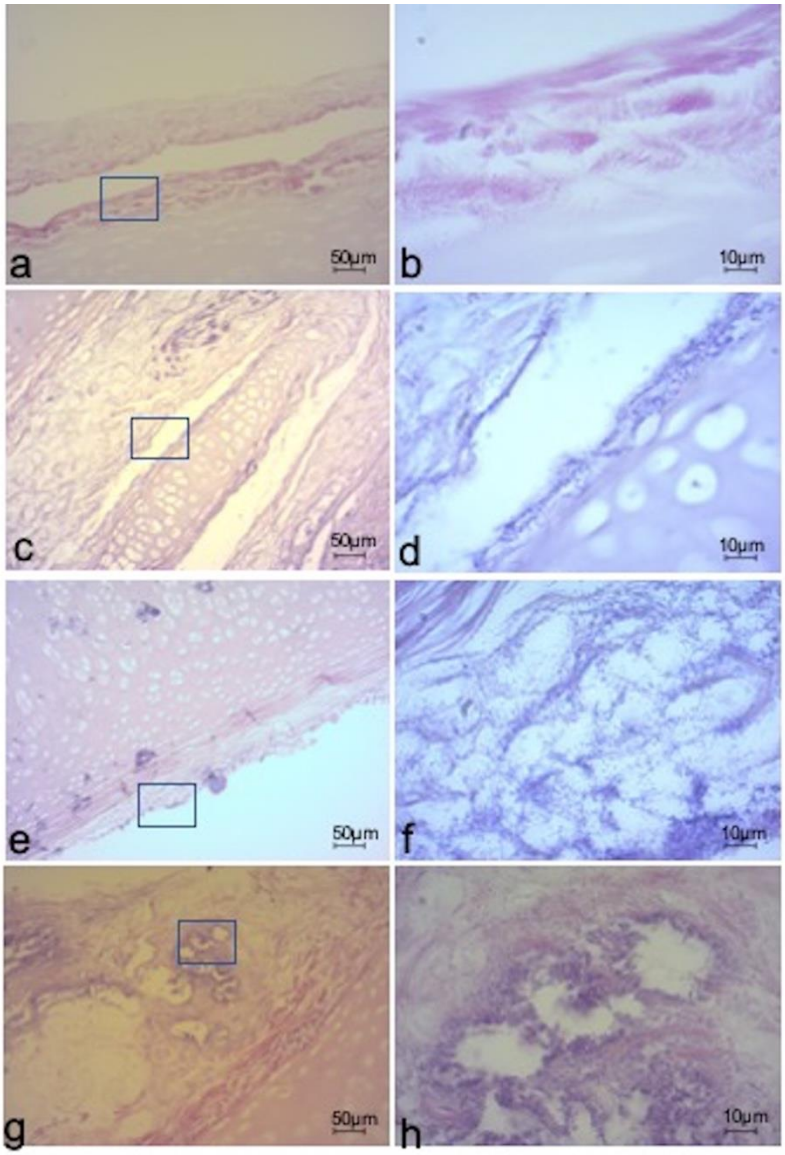


Figure S4. H & E stain of representative lungs at day 7.
Uninfected control (a,b) and 7 days post inoculation with FQ151 (c, d) FQ184 (e, f) and USA300 (g,h). x20 magnification (a,c,e and g). Highlighted area of interest (rectangle) is then shown at x100 magnification (b, d, f and h). Scale bars represent 50µm and 10µm accordingly. Clusters of cocci shaped cells are present at or near tissue airway interface for all inoculated tissue at x 100 (d, f and h) but not uninfected control (b).

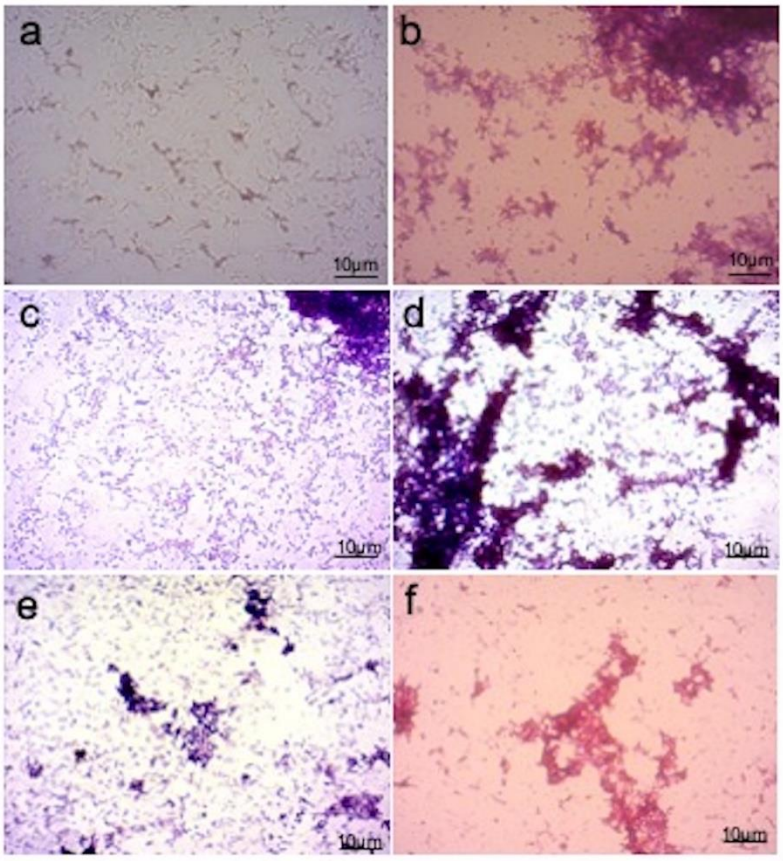


Figure S5. Gram stain of ASM samples surrounding lung at 7 day post inoculation in EPVL.

Uninfected control (a,) and 7 day post inoculation with USA300LAC (b), FQ128 (c) FQ140 (d), FQ142 (e) or FQ151 (f) magnification x100, scale bar represents 10µm. Aggregations of gram positive cocci, are seen in infected samples (b-f) but not uninfected control. Larger aggregates are observed in biofilm forming strains (b,c, d and f). Tissue and endogenous or contaminate bacteria are visible (but poorly stained) in uninfected control.

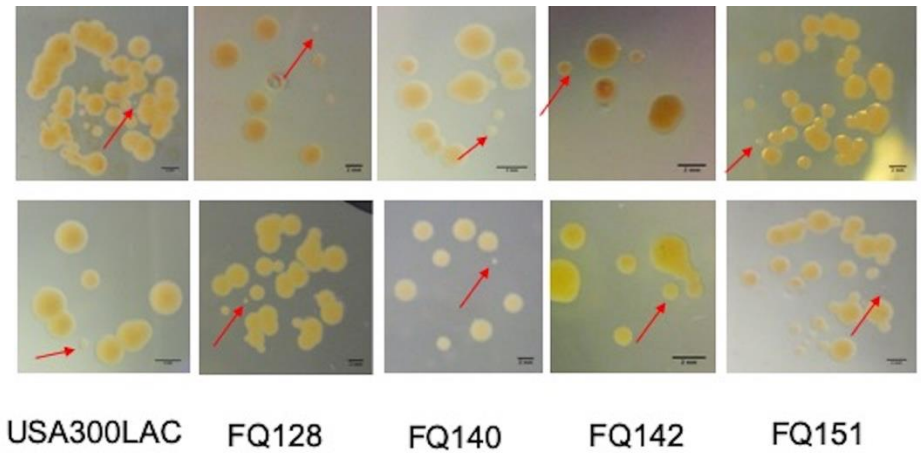


Fig S6. Appearance of SCV on MSA plates. Samples taken from lung homogenate (top line) or surrounding ASM (bottom line) at 7 days post inoculation and incubated for 24 H at 37ºC in 5% CO_2 and a further 48 H in ambient conditions. Red arrows indicate typical SCV, selected and identified by catalase and coagulase tests and by PCR (Table S2).

Table S2. Identification of Small Colony Variants (SCVs). Catalase and coagulase result and colony identity as confirmed by 16S sequence. Samples were taken from lung homogenate or surrounding Artificial Sputum Media (ASM) at 7 days post inoculation and grown on MSA plates for 24 h at 37 °C in 5% CO_2 and a further 48 h in ambient conditions. SCVs were selected as shown in Fig S1 and all confirmed with *Staphylococcus* specific primers prior to sequencing of the 16S-23S intergenic spacer.

Strain originally inoculated	Diameter of small colony identified (% WT)	Coagulase	Catalase	16S sequence identity
USA300	27	+	+	*S. aureus*
USA300	32	+	+	*S. aureus*
FQ128	31	+	+	*S. aureus*
FQ128	23	+	+	*S. aureus*
FQ140	26	Weak	+	*S. aureus*
FQ140	25	Weak	+	*S. aureus*
FQ142	43	+	+	*S. aureus*
FQ142	43	+	+	*S. aureus*
FQ151	40	Weak	+	*S. aureus*
FQ151	31	Weak	+	*S. aureus*