
Supplementary information

**Tolerance and resistance of microbial
biofilms**

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Supplementary information:

Supplementary figure 1:

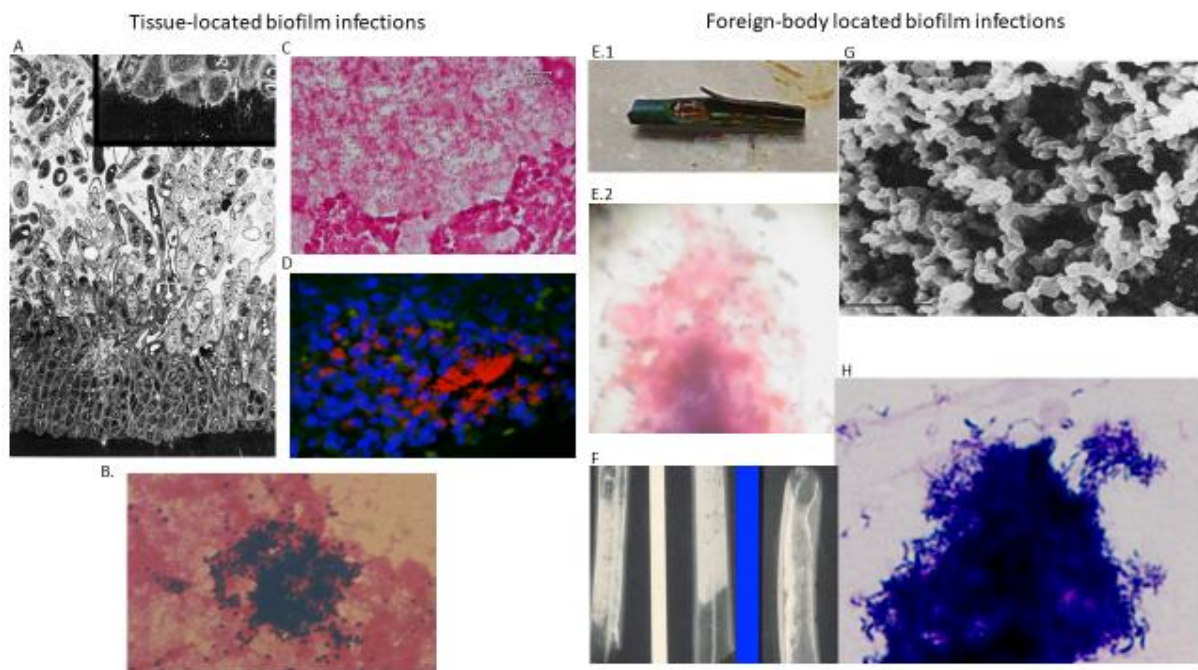


Figure S1. **Representative macroscopic and microscopic pictures of biofilm-related infections.** Panels A-D show tissue-located biofilms, and panels E-H show foreign body-located clinical biofilm infections. Panel A is an electron microscopy (EM) picture of dental plaque, a tissue surface-related biofilm. In addition, dental plaque represents a mixed biofilm with the involvement of several different bacterial species. The biofilm is formed by bacterial adhesion on the pellicle, with streptococci and *Actinomyces* spp. forming the densely packed basis. Further biofilm colonization includes diverse bacterial species with a looser structure, which can also be seen in the EM image ¹. Panel B shows a Gram-stained sample from an aortic valve showing aggregated *S. aureus* which are surrounded by amorphous material represented by fibrins, thrombocytes and cell debris. The initial colonization is made possible by microscopic damage to the endothelial layer of the heart valves and subsequent formation of sterile fibrin and thrombocyte vegetation. Panel C shows a Gram-stained specimen from an explanted lung of a 41 year old cystic fibrosis patient who had suffered from chronic lung *P. aeruginosa* infection for 28 years and had been treated with 114 antibiotic courses, illustrating the survival of large biofilm of *Pseudomonas aeruginosa* with matrix material attracting polymorphonuclear neutrophils, especially on the

left side of the picture (by courtesy of prof. Niels Høiby). Panel D shows a biopsied sample obtained from a non-healing wound and showing aggregates of *P. aeruginosa* stained and identified by means of a specific peptide-nucleic acid-fluorescence in situ hybridization (PNA-FISH) technique (red stain) surrounded by host cells (DAPI blue stain) ². Panel E1 shows a bile stent from a patient with fatal relapse of Gram-negative sepsis. Panel E2 shows Gram-stained biofouling (biofilm formation on surfaces) of the internal surface of the stent removed post-mortem from the patient. The material contains aggregates of *Escherichia coli* and *Klebsiella oxytoca* ³. Panel F shows an X-ray image of an endotracheal tube revealing biofouling and on the verge of being pushed into the airways of the patient, resulting in ventilator-associated pneumonia (VAP) ⁴. Panel G shows an EM image of aggregated *S. aureus* on a cardiac pacemaker electrode from a patient with relapsing *S. aureus* bacteraemia. The bar represents 5 μm ⁵. Panel H demonstrates a large aggregate of *Cutibacterium acnes* (stained by methylene blue) released from a cerebral ventricular-atrial shunt inserted in a patient with compromised cerebrospinal fluid drainage (Panels B, C and H kindly provided by Niels Høiby, University of Copenhagen, Denmark).

Panel A, reprinted with permission from Ref. 1, FADL's Forlag A/S.

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Supplementary Box 1: Principles for treating biofilm infections

Since biofilm infections induce innate inflammation, which is aggravated by the adaptive immune response, tissue damage around biofilms always occurs (Figure 1). Furthermore, under certain conditions, biofilm infections may be foci for the spread of pathogens to the blood and/or organs. Biofilm infections are, therefore, diseases that should be prevented by hygienic measures (e.g., tooth brushing), antibiotic prophylaxis or pre-

emptive treatment, treated and cured with antibiotics, removed mechanically (in the case of foreign bodies) or suppressed with antibiotics⁶.

All the different principles of antibiotic prevention and treatment of microbial infections are therefore used in biofilm infections. Prophylactic use to prevent biofilm infections is widely used in orthopaedic surgery when artificial joints are implanted to replace arthritic joints ⁶.

Pre-emptive treatment of colonization before it develops to chronic biofilm lung infections is a universally used approach in cystic fibrosis patients intermittently colonized with *P. aeruginosa*^{6, 7, 8}

Empiric treatment of suspected biofilm infection is sometimes the best approach when cultures are not obtainable due to the risks posed by the sampling procedure, e.g., mycotic aortic aneurism or pancreatic stent infections. In these cases, blood cultures may guide the choice of antibiotics ⁶

Definitive treatment is based on culturing the offending microorganism, but in contrast to planktonic infections, antibiotic susceptibility testing, although used to guide the choice of antibiotics, does not lead to the cure of many biofilm infections, with the exception of endocarditis treated with high dose for a prolonged period ^{6,9}

Chronic suppressive antibiotic therapy to maintain the function of the infected organ, e.g., the lungs of cystic fibrosis patients with *P. aeruginosa* biofilm infection, is universally used ⁷.

For example, the principle of chronic suppressive antibiotic therapy for cystic fibrosis patients with chronic *P. aeruginosa* biofilm infections in the lungs is based on local antibiotic therapy administered for years through daily inhalation of colistin, tobramycin, aztreonam, levofloxacin and additional intravenous antibiotics are administered regularly every 3 months, or intravenous antibiotics are administered for 2-3 weeks to treat acute exacerbated infection in the respiratory zone of the lungs ^{7,10}. Inhaled antibiotics reach the conductive airways of the lungs (trachea, bronchi, bronchioles, and terminal bronchioles) harbouring bacterial biofilms in sputum, and the intravenous antibiotics reach the respiratory airways (alveoli, alveolar sacs, and respiratory bronchioles) where biofilms are developed during acute exacerbations, which destroy pulmonary lobules and further reduce lung function ¹¹. Similarly, local treatment with antibiotics in fibrin clots after sinus surgery for *P. aeruginosa* biofilm sinusitis is needed, since systemically delivered doses generally do not provide sufficient concentrations in the paranasal sinuses where remaining biofilms may remain after surgery ¹². Local flushing of the sinuses with antibiotics is not efficient since not all paranasal sinuses can be flushed and even the maxillary sinuses are quickly emptied of the antibiotics¹³.

Supplementary Box 2: The role of antimicrobial susceptibility testing in biofilm treatment

Antibiotic susceptibility tests in clinical microbiology laboratories are either performed as broth dilution tests in microtiter wells or as agar diffusion tests in solid agar plates. Modifications of the dilution method have been published for biofilm use, e.g., the Calgary Biofilm Device¹⁴. The method depends on the formation of a biofilm attached to an artificial surface, e.g., the pegs of the lids of a microtiter plate. When the biofilm is formed after 18-24 hrs (young biofilms) or longer (old biofilms), the lid with the pegs is removed and placed in another microtiter plate with increasing concentrations of antibiotics in the wells, and after exposure to the antibiotics for 18-24 hrs, the lid with the biofilms is washed and placed in another microtiter plate with antibiotic-free media, sonicated and growth in the different wells is observed after 18-24 hrs.¹⁵ The lowest concentration of an antibiotic that has killed the biofilm and thereby prevented growth is called the minimal biofilm eradication concentration (MBEC), which increases with the age of the biofilm¹⁶. The problem with this test is that it investigates large attached biofilms visible to the naked eye, whereas non-attached biofilms in the tissue are much smaller (5-100 μm), similar to the young biofilms, compared to attached biofilms, which may be >1000 μm ; therefore, old biofilms may be more resistant¹⁷ and that some clinical isolates might not form biofilms on the plastic pegs. Agar diffusion tests provide useful information on biofilm tolerance to antibiotics since the formation of the inhibition zone during the agar diffusion antibiotic susceptibility test is caused by a switch from the planktonic to the biofilm mode of growth and therefore provides clinically important information on the seeded planktonic bacteria that are susceptible, but biofilms of the inhibition zone are resistant due to the antibiotic tolerance of biofilms¹⁶.

Since no in vitro biofilm antibiotic susceptibility tests are approved by EUCAST or CLSI, the choice of antibiotics for the treatment of biofilm infections is based on standard susceptibility testing of inocula of planktonically growing bacteria. This is appropriate when dealing with prophylaxis and pre-emptive use of antibiotics to prevent biofilm infections. When choosing antibiotics to treat established biofilm infections, the reason to use planktonic antibiotic susceptibility results is to detect mutational or horizontally transferred resistance mechanisms, such as beta-lactamase production, which, as discussed previously, further prevents the activity of antibiotics on biofilm infections. Although not eliminated by antibiotics, biofilms are reduced in size, the number of CFUs is reduced and inflammation is reduced during antibiotic therapy for biofilm infection, e.g., CF patients with chronic *P. aeruginosa* biofilm lung infections¹⁰. Genetically multiresistant strains of biofilm-

growing *P. aeruginosa* have been previously shown to spread quickly between CF patients treated in centres^{18,19}.

Supplementary Box 3: Antimicrobial resistance in biofilms

The development of mutational antibiotic resistance in biofilms with established antibiotic tolerance may seem unnecessary from an evolutionary point of view. However, the development of genetic resistance reinforces the recalcitrance of infectious biofilms, and it has been shown that resistance of *P. aeruginosa* populations due to hyperexpression of AmpC beta-lactamase or MexAB-OprM efflux pumps protect the whole bacterial community, preserving susceptible populations from the action of antibiotics²⁰. In vitro studies of mixed populations of resistant and sensitive bacteria showed that genetically resistant mutants are situated in the surface layer of a biofilm, shielding the remainder of the biofilm population from the action of antibiotics. The development of AMR in biofilms allows bacterial proliferation, and bacterial cells with an AMR phenotype that detach from the biofilm have the potential to initiate new recalcitrant biofilms, combining the tolerance of the bacteria in a biofilm mode of growth with the resistance of the bacterial population that initiate growth.

Mutators (G) are frequently isolated from biofilm samples taken from CF patients with chronic lung infection, patients with chronic obstructive lung disease (COPD) and people with chronic urinary tract infections²¹⁻²⁵ despite the generally accepted fact that mutator phenotypes are self-limiting due to the high risk of deleterious mutations²⁶. The ability to acquire these mutators suggests that they are adapted to and maintained in some biofilm niches to survive intense antibiotic treatment and inflammation. Mutators probably compensate for slow bacterial growth by ensuring a pool of spontaneous mutants that allow adaptation of the biofilm and emergence of resistance in biofilms. Enhanced adaptability of mutants compared to the wild-type *P. aeruginosa* was observed when grown in competition experiments in flow-cell biofilms^{27,28} and correlates with the long-term clinical observations of *P. aeruginosa* mutators in CF lungs²⁹.

Fast development of resistance to phages due to mutation in the molecules used by phages to adhere to the bacterial cell, such as LPS, type IV pili or outer membrane proteins during repeated treatments, is a major challenge for the implementation of phage therapy, and a combination of various phages and likely a combination of antibiotics will be required to overcome it³⁰.

Supplementary Box 4: Strategies for treating biofilm infections

Targeting the matrix

Targeting the matrix would destabilize the biofilm structure, paving the way for eradication of the infection by the immune system and would improve the penetration of the antimicrobial molecules in the biofilm. Methods to dissolve the biofilm matrix have been shown to work in vitro and in animal experiments but have not yet been used in the clinic^{31, 32,33}. A phase 2 clinical study with an alginate oligosaccharide with biofilm-disrupting properties (Oligo G) in patients with cystic fibrosis (NCT03822455) is in progress. DNase is used routinely in the treatment of chronic *P. aeruginosa* lung infection in CF patients to destabilize eDNA, the main source of which is destroyed PMNs³⁴. Alternatively, enzymes cause depolymerization of the matrix or activation of natural dispersal mechanisms^{33, 35,36}. Dispersin B, a glycoside hydrolase with biofilm-dispersing activity, inhibits biofilm formation of *S.aureus*, *S.epidermidis* and *E.coli* and disperses *S.epidermidis* and *E.coli* biofilms. Low-dose nitric oxide administration was shown to stimulate *P. aeruginosa* biofilm dispersal through a decrease in c-di GMP levels.³⁷ Recently, small molecules that inhibit c-di GMP, able to disperse *P.aeruginosa* biofilms have been identified³⁸. Like all agents stimulating biofilm dispersal, matrix degrading molecules would need to be combined with antibiotics to prevent spread of the bacteria to other sites in the human body. Acquisition of right concentration of matrix dispersing agents and antibiotics at the site of infection might be a challenge for the translation of this strategy to the clinic³⁹.

Targeting the heterogeneity /persister cells in biofilm populations

Since the low metabolic state of the bacteria is a common feature of tolerant bacterial biofilm populations, modulating metabolic activity is a possible approach for improving the efficacy of bactericidal antibiotics. However, these strategies have not been tried with biofilm-growing bacteria, although they have been shown to work in stationary-phase cells, e.g., aminoglycosides combined with TCA cycle metabolites and ciprofloxacin combined with glucose and fumarate⁴⁰.

Several strategies to fight persister cells have been proposed and grouped into three categories: killing metabolically dormant persister cells circumventing the need for active cell processes, sensitization to antimicrobials by resuscitation of persister cells and interference of the formation of persister cells, as described in a comprehensive review⁴¹.

In vivo, these strategies would theoretically increase the risk of spreading the infection, which can be a matter of concern in immunocompromised patients requiring simultaneous aggressive antibiotic therapy with bactericidal compounds.

Oxygen as enhancer of antibiotic activity

The improved outcome of antibiotic treatments of several types of biofilm infections by supplemental O₂ supports the fact that the microenvironment of biofilms is microaerophilic or anaerobic and therefore less susceptible to antibiotics⁴². Biofilm infections that may soon become susceptible to antibiotics by oxygenation include endocarditis⁴³, osteomyelitis, brain abscesses and device-related infections⁴².

Hyperbaric oxygen treatment is already used in the clinic for treatment of clostridial infections and refractory chronic wounds but application as adjuvant anti-biofilm therapy requires further research to establish the right dosis and duration of treatment required for the different types of biofilm infections⁴⁴.

Anti-evolutionary drugs

As the production of mutagenic ROS and the activation of the SOS response are involved in increased mutagenesis in biofilms with an increased risk of AMR development, drugs that inhibit these mechanisms, so-called anti-evolutionary drugs, might prevent the development of resistance in biofilms^{45,46}. ROS-reducing drugs (antioxidants), such as edaravone, have been shown to reduce the evolution of AMR in ciprofloxacin-treated *E. coli* populations⁴⁵

Anti-inflammatory treatments targeting pathogenesis of biofilm infections

Since tissue damage around biofilm infections is due to the inflammatory response, immune suppression by local or systemic steroid or non-steroid anti-inflammatory drug treatment has been shown to be effective, but side effects limit their long-term use^{47,48}. The effect of azithromycin therapy on chronic *P. aeruginosa* lung infection in CF has also been shown to involve anti-inflammatory activity⁴⁹. For chronic wounds, application of autologous LeucoPatches (3C Patch[®], Reapplied, Birkerød, Denmark), consisting of fibrin, thrombocytes and leucocytes⁵⁰, improved PMN phagocytic and burst activity against biofilm-growing *P. aeruginosa*⁵¹, and in a randomized controlled study, it was shown to accelerate the healing of diabetic, hard-to-heal foot ulcers⁵². Topical application of S100A8/A9 to re-establish its pre-suppression levels in chronic wounds enhanced the killing of biofilm-growing *P. aeruginosa*⁵³, acted in synergy with systemic ciprofloxacin and prevented the

development of resistance to ciprofloxacin⁵⁴ in a mouse chronic wound model. The background for this treatment is the PMN-related alarmin Ca^{+++} -binding heterodimer S100A8/A9 (calprotectin), which is normally found to be increased during infection and has been reported to be suppressed in non-healing wounds, compared to its level in healing wounds⁵⁵, due to the presence of *P. aeruginosa* biofilms⁵⁶. The mechanism of biofilm suppression of calprotectin is not understood; however, animal experiments have shown that the presence of *P. aeruginosa* biofilm infection results in the suppression of two additional PMN-related cytokines, granulocyte colony-stimulating factor and keratinocyte-derived chemokine (KC, a murine IL-8 analogue). The suppressed calprotectin levels, the resulting reduced antimicrobial effect and the changed immune-modulating effects probably add to the pathogenicity of biofilm infections⁵⁷.

A Th2-dominated immune response leading to a pronounced antibody response has been shown to correlate with the poor prognosis of chronically infected CF patients^{58,59}. A Th1-dominated immune response (downregulation of the humoral response) correlated with better lung function in CF⁶⁰. Although a change from a Th2-dominated to a Th1-dominated immune response has been shown to be beneficial in animal studies, a clinical trial with inhalation IFN- γ failed to demonstrate a beneficial effect in chronically infected CF patients⁶¹. To our knowledge, direct modulation of the adaptive immune response has not been realized against biofilm infections.

Improved PK/PD of antibiotics in biofilms

High antibiotic concentrations must be administered to biofilms for prolonged periods, and systemic toxic side effects (such as nephro- or hepatotoxicity) may therefore be induced unless local application of antibiotics can replace or can be added to systemic antibiotics.

These principles of local administration of antibiotics to treat biofilm infections have also been used extensively for the treatment of chronic osteomyelitis, ventriculitis in hydrocephalus shunt patients, infections in intravenous lines and CF¹⁶. An example is the treatment of chronic, biofilm-related lung infection in CF where inhalation of antibiotics results in achievement of substantially higher antibiotic target site concentration in the lung secretion than could have been achieved by systemic administration. Thereby, decreased drug susceptibility, as evaluated using planktonic susceptibility breakpoints is overcome, albeit only in the upper airways¹⁶. Similarly, it has for many years been routine to install local antibiotic as e.g. gentamicin loaded beads or other carriers during revision of orthopaedic prosthesis revision, and thereby attaining substantially higher antibiotic concentrations at the site of infection, which could not have been obtained through systemic

admission without significant toxic side effects⁶. For colonized central venous catheters which cannot always be removed or replaced during the acute situation, antibiotic or non-antibiotic (ethanol or HCl) lock therapy strategy in parallel to relevant systemic antibiotic therapy is often used ⁶.

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