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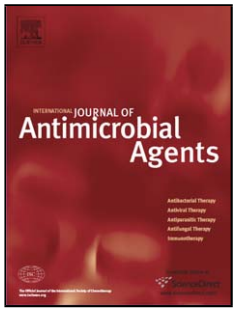

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Antibiotic resistance of bacterial biofilms

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ABSTRACT

A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics, desinfective chemicals and resist phagocytosis and other components of the defense system of the body. The persistence of e.g. staphylococcal infections related to foreign bodies is due to biofilm formation. Likewise, chronic *Pseudomonas aeruginosa* lung infections in cystic fibrosis patients are caused by biofilm growing mucoid strains.

Characteristically, gradients of nutrients and oxygen exsist from the top to the bottom of biofilms and these gradients are associated to decrease of bacterial metabolic activity and increase of doubling times of the bacterial cells and these more or less dormant cells are therefore responsible for some of the tolerance to antibiotics.

Biofilm growth is associated with increased level of mutations and with quorum sensing regulated mechanisms. Conventional resistance mechanisms such as chromosomal beta-lactamase, up-regulated efflux pumps and mutations of antibiotic target molecules in the bacteria also contribute to the survival of biofilms. Biofilm can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppresive therapy. A promising strategy may be the use of enzymes which can dissolve the biofilm matrix (e.g. DNase, alginate lyase) and quorum sensing inhibitors, which increases biofilm susceptibility to antibiotics.

1. Introduction

Biofilm growing bacteria cause chronic infections (1) which are characterized by persisting inflammation and tissue damage (2). Chronic infections, including foreign

body infections, are infections which 1) persist in spite of antibiotic therapy and the innate and adaptive immune- and inflammatory response of the host, and 2) which, in contrast to colonization, are characterized by immune response and persisting pathology (Table 1).

2. The occurrence and achitecture of bacterial biofilms

Foreign body infections are characterized by biofilm growth of the bacteria on the outer and/or inner surface of the foreign body (Table 2). Biofilm growth also occurs on natural surfaces such as teeth (3), heart valves (endocarditis), (4) in the lungs of cystic fibrosis (CF) patients causing chronic bronchopneumonia (2), in the middle ear in patients with persistent otitis media (5), in chronic rhinosinusitis (6), in chronic osteomyelitis and prostetic joint infections (7) (8) (9), in intravenous catheters and stents (10) and in chronic wounds (11) (12)(Fig. 1 A & B). The microbes in biofilms are kept together by a self-produced biopolymeric matrix. The matrix contains polysaccharides, proteins and DNA originating from the microbes and the bacterial consortium can consist of one or more species living in a sociomicrobiological way (1, 13) (2, 14). The matrix is important since it provides structual stability and protection to the biofilm. The development of bacterial biofilms over time has been intensively studied *in vitro* by confocal scanning laser microscopy employing *green fluorescent protein (gfp)*-tagged bacteria. This technique has been combined with advanced *in silico* image-analysing to produce 3-D images of biofilm (15, 16) (17). As an example, *P. aeruginosa* produce a mature *in vitro* biofilm in 5-7 days (Fig. 2).

The development of an *in vitro* biofilm is initiated by planktonic (freely moving) bacteria which are attached reversible to a surface, which may be conditioned of a layer of e.g. proteins (a pellicle) (3) (18). At this stage, the bacteria

are still susceptible to antibiotics and this is in accordance with the success of the peroperative antibiotic prophylaxis for e.g. alloplastic surgery. The next step is irrreversible binding to the surface within the next few hours and multiplication of the bacteria which forms microcolonies on the surface and begin to produce a polymer matrix around the microcolonies (18) . The biofilm grows thickness (up to 50 µm) and under *in vitro* conditions, mushroom-like or tower-like structures are often observed in the mature biofilm. At that stage, the biofilm show maximum tolerance (= resistance) to antibiotics. Subsequently follows a stage where focal areas of the biofilm dissolves and the liberated bacterial cells can then spread to other location where new biofilms can be formed. This liberation process may be caused by bacteriophage activity within the biofilm (19). The mature biofilm may contain water-filled channels and thereby resemble primitive, multicellular organisms. Motile bacteria can use type-1V pili to mount or climb a biofilm formed by other bacteria and colonize the top of the biofilm resembling a hat (16). Important properties of biofilm growing bacteria are different from those of planktonic growing bacteria and this has significant diagnostic and therapeutic consequenses. The bacteria appear different in biofilm infections since they are located close to each other in aggregates surrounded by the self-produced matrix. In clinical specimens (biopsies, pus, sputum) biofilm can often be recognized by light microscopy although precise identification of all the bacteria within a biofilm can only be done by DNA hybridization techniques and identification of the components of the biofilm matrix require specialized staining techniques (2). Traditional sampling techniques may not be suffucient to culture biofilm growing bacteria sticking to a surface unless the bacteria are released by ultasonic pre-treatment (20). The ordinary culture techniques, however, reveals only the properties of planktonically growing bacteria, and e.g. antibiotic susceptibility

testing therefore gives misleading results which do not reflect the increased resistance of the bacteria when living in biofilms. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to biofilm growing bacteria may be up to 100 – 1000 fold higher compared to planktonic bacteria (21) (22, 23). Methods to test biofilm growing bacteria have therefore been developed but their clinical relevance as regards prediction of clinical successfull therapy awaits confirmation (22, 24, 25).

3. Stationary phase physiology, low oxygen concentration and slow growth.

Inspection of environmental as well as in vitro biofilms has revealed that oxygen concentration may be high at the surface but low in the center of biofilms where anaerobic conditions may be present (26). Likewise, growth, protein synthesis and metabolic activity is stratified in biofilms i.e. high level of activity at the surface and low level and slow growth or no growth in the center and this is one of the explanations for the reduced susceptibility of biofilms to antibiotics (27) (28). Very slow *in situ* growth rates of biofilms of *P. aeruginosa* have been measured in sputum of CF patients (average doubling time 2-3 hours and presence of a significant number of cells in stationary growth fase) (29). Monotherapy with antibiotics like beta-lactams, which are only active against dividing *P. aeruginosa* cells, are therefore not very efficent to eradicate biofilm infections (21).

4. Mutators

The mutation frequency of biofilm-growing bacteria is significantly increased compared to planktonically growing isogenic bacteria (30) and there is an increased horizontal gene transmission in biofilms (31). These physiological conditions may explain why biofilm growing bacteria easily become multi-drug resistant by means

of traditional resistance mechanisms against beta-lactam antibiotics, aminoglycosides and fluroquinolones which are detected by routine susceptibility testing in the clinical microbiology laboratory where planktonic bacterial growth is investigated. Thus, bacterial cells in the biofilms may simultaneously produce enzymes that degrade antibiotics, have antibiotic targets of low-affinity and overexpresses efflux pumps which have a broad spectrum of substrates. Achievement of multiple mutations in a bacterial population size of 10^8-10^{10} / ml sputum as is attained under infection of the CF lung (32) imply the presence of a hypermutable bacterial subpopulation and the presence of high percentages of hypermutable *P. aeruginosa* isolates associated with antibiotic resistance have actually been found in CF patients (33) (34).

The hypermutable phenotype of CF *P. aeruginosa* isolates is due to alterations in the genes of the DNA repair systems of either the mismatch repair system (MMR) which involves *mutS, mutL* and *uvrD* or of the DNA oxidative lesions repair system (GO) which involves *mutT, mutY* and *mutM* (35, 36). It has been shown that mutations in either of the two systems determine emergence of antibiotic resistant isolates, especially due to selection of isolates expressing multidrug efflux-pumps (36, 37). An increased production of endogenous reactive oxygen species and a deficient anti-oxidant system (38) (39) determine an imbalance between oxidative burden and anti-oxidant defenses leading to oxidative stress in biofilms. The oxidative stress is considered to cause enhanced mutability in biofilms (30, 40) . Recent data suggest that microcolony structures, due to endogenous ocidative stress, are specific sites within the biofilms for enhanced genetic adaptation and evolutionary change (40). In addition, Boles and Singh (41) showed that the endogenous oxidative stress in biofilms promote antibiotic resistance and that addition of anti-oxidants reduced the

occurrence of diversity in biofilms. We have previously shown that oxidative stress is linked to the occurrence of hypermutable *P. aeruginosa* strains in CF patients (34).

In addition to their endogenous oxidative stress, the biofilm-growing bacteria in the CF airways are exposed to reactive oxygen species (ROS) from the activated polymorphonuclear leukocytes (PMN)(34). We have recently shown that the hypoxic environment in the CF sputum is due to the consumption of oxygen by PMNs which liberate reactive oxygen species which can react with the biofilm-embedded bacterial cells (42), thus creating an unique environment with low-oxygen tension filled with reactive oxygen species.

The hypermutability of bacteria in biofilms promotes the emergence of mutations conferring antibiotic resistance which will be selected for by the repeated antibiotic courses administered in order to maintain the lung function of CF patients. Development of resistance to all classes of antibiotics during the chronic lung infection in CF has been documented (43). Resistance to beta-lactam antibiotics occur due to mutations in regulatory genes of the beta-lactamase production leading to the occurrence of isolates with stable or partially-stable derepressed production of AmpC beta-lactamase(44). Resistance to ciprofloxacin of CF *P. aeruginosa* isolates was shown to be mediated by mutations in *gyrA* and alterations in the two efflux systems MexCD-OprJ and MexEF-OprN and resistance to tobramycin was overexpression of the MexXY-OprM multi-drug efflux pump (45) (46). Resistance to colistin was shown to occur due to mutations in the *pmr* system involved in the LPS structure (47).

5. Chromosomal beta-lactamase and biofilm matrix components

The overproduction of chromosomally encoded AmpC cephalosporinase is considered the main mechanism of resistance of CF isolates of *P. aeruginosa* to β-lactam antibiotics (48). The most common phenotype of beta-lactamase production in CF isolates is the partially derepressed phenotype with high basal levels of beta-lactamase that can be induced further to higher levels in the presence of beta-lactam antibiotics (44). The role of this beta-lactamase phenotype is important especially for the resistance to beta-lactam antibiotics acting as strong inducers (carbapenems like imipenem). However, not all beta-lactams are strong inducers and the overexpression of the MexAB-OprM efflux pumps may play, together with beta-lactamases, an important role in the resistance to poor inducers (e.g. piperacillin). Totally derepressed beta-lactamase production is encountered in 2.5% of clinical CF isolates (44) and is responsible for the resistance to both poor and strong inducer beta-lactam antibiotics, independent of the presence of efflux pumps overexpression (49) (50)(51). We have found an insertion sequence (IS 1669) inactivating the *ampD* gene in several resistant clinical *P. aeruginosa* isolates with constitutive high expression of chromosomal beta-lactamase (51) .

The diffusion barrier in biofilms (52) plays a role for biofilm resistance of *P. aeruginosa* that overproduce beta-lactamase due to the presence in the biofilm matrix of beta-lactamases which will hydrolyze the beta-lactam antibiotics before reaching the bacterial cells (53) (54). Giwercman (55) showed that imipenem and piperacillin were able to induce β-lactamase production in *P. aeruginosa* biofilms. Nichols (56) predicted from mathematical models that the biofilm would not afford protection

against diffusion of beta-lactam antibiotics into the bacteria embedded in the biofilm as long as the level of chromosomal beta-lactamase is low. However bacteria expressing high level of chromosomal beta-lactamase growing in biofilms would be exposed to reduced concentration of beta-lactam antibiotics due to accumulation of the enzyme in the polysaccharide matrix. The extracellular β-lactamase would inactivate the antibiotic as it penetrates, thereby protecting the deeper-lying cells.

The source of β-lactamase in biofilms has been considered to be from a layer of lysed bacteria due to exposure to an antibiotic, with release of defensive enzymes into the extracellular space. We have shown that the source of β-lactamase in biofilm may also be the membrane vesicles (MVs) containing beta-lactamase liberated by resistant *P. aeruginosa* bacteria (57)(58)(54) and we have shown, that high level of free chromosomal beta-lactamase is present in CF sputum (58). We have also shown that strong inducers like imipenem will induce the beta-lactamase through all the bacterial layers while poorer inducers like ceftazidime will influence just the superficial layers of the biofilm, probably due to the inactivation of the antibiotic by beta-lactamase (59, 60)(61)(58) (Figure 3).

The protective role played by beta-lactamase in impairing the penetration of beta-lactams in the biofilm can be seen in Fig. 3A. Treatment with ceftazidime of a biofilm formed by a *P. aeruginosa* CF strain with stable derepressed levels of beta-lactamase due to an insertion sequence in *ampD* (*P. aeruginosa ampD*$^-$) killed very few bacterial cells (dead bacteria in red) (Figure 3B) in contrast to the complemented strain with low level of beta-lactamase (Fig. 3 C). However, addition of aztreonam improved the efficacy of ceftazidime treatment of the biofilm (Figure 3D, probably because aztreonam acts as a beta-lactamase inhibitor (58). In addition, meropenem, a

beta-lactamase stable beta-lactam showed good *in vitro* efficacy in the treatment of *P. aeruginosa* biofilms (22) (61). Treatment with ceftazidime of a biofilm formed by the same strain expressing basal levels of beta-lactamase due to complemention with the wild-type *ampD (P. aeruginosa ampD+*) led to eradication of the biofilm (Figure 3C).

The matrix of the biofilm may also be part of the resistance mechanisms to antibiotics since e.g. sub-MIC concentrations of beta-lactam antibiotics induce increased alginate synthesis in *P. aeruginosa* biofilms (Fig. 4) (62) (63) and also enhance the biofilm matrix of some slime-producing coagulase-negative staphylococci (64)(65). Originally, it has been thought that tolerance of the biofilms to aminoglycosides was due to a transport limitation due to the binding of these positively charged antibiotics to the negatively charged exopolysaccharide matrix but the repeated dosing of antibiotics during therapy probably leads to saturation of the binding sites (66) (67) (68). As previously discussed, the oxygen limitation and the metabolic rates are probably more important factors which contribute to the tolerance of biofilms to aminoglycosides and ciprofloxacin (69). However, in the respiratory zone of the CF lung with poor access to aminoglycoside aerosols, where the antibiotic concentration is low delayed penetration of the aminoglycosides through thick biofilms may play a role in the tolerance of biofilms to aminoglycosides (2). Recently, it has been shown that administration of DNase and alginate lyase enhanced the activity of tobramycin in biofilms by dissolving the biofilm matrix (70).

6. Tolerance, adaptive resistance and efflux pumps

Colistin is only active against the non-dividing central part of *P. aeruginosa* biofilms *in vitro* (Fig. 5A), whereas the superficial, metabolic active part of the biofilm

become tolerant due to upregulation of PmrA-PmrB two-component regulatory systems involved in the adaptive resistance to cationic peptides leading to addition of aminoarabinose to lipid A of LPS (22, 71, 72). Since the metabolic active surface layer of the biofilm is susceptible to ciprofloxacin (Fig. 5B) in contrast to the dormant central part of the biofilm, combination therapy with this drug and colistin was able to kill all cells in the biofilm *in vitro* (Fig. 5C)(71). The clinical efficacy has been demonstrated of this combination therapy for the early eradication treatment of *P. aeruginosa* in CF patients (73).

Tolerance of biofilms to tobramycin is also mediated by low metabolic activity but the high cell density that results in accumulation of extracellular signalling molecules is probably important, as it has been shown that tolerance to tobramycin of *P. aeruginosa* strain PAO1 biofilm is quorum sensing mediated (Fig. 6)(74) (see below). In addition, a non-specific mechanism for the tolerance of the metabolic active cells to colistin was shown to be up-regulation of the efflux pump MexAB-OprM (71). Furthermore, increased efflux pump activity due to mutations has been shown to be a major resistance mechanism against aminoglycoside antibiotics and fluorquinolones in *P. aeruginosa* from CF patients (46) (45).

7. High cell density and quorum sensing

Bacteria communicate by means of synthizising and reacting on signal molecules (75-78). The term QS indicates that this system permits bacteria to sense when a critical number (concentration) of bacteria are present in a limited space in the environment and respond by activating certain genes which then produce e.g. virulence factors such as enzymes or toxins. The QS molecules are small peptides in many Gram-

positive bacteria whereas the most well-described QS molecules in Gram-negative bacteria are N-acyl-L-homoserinelactones (AHL) (78). For *P. aeruginosa* QS regulates the production of virulence factors such as extracellular enzymes and cellular lysins (e.g. rhamnolipid), which are important for the pathogenesis of infections where it functions as a protective shield against phagocytes (79, 80) (81). QS may also have influence on the development of the biofilm (82) and QS have been shown to determine the tolerance of *P. aeruginosa* biofilms against antibiotic therapy and against the innate inflammatory response dominated by polymorphonuclear leucocytes (PMNs) (83). The connections between QS and biofilms has been named sociomicrobiology (84).

8. Quorum sensing inhibitors (QSI)

Much of our knowledge about QS orginate from experiments with QS knock-out mutants and from use of naturally occurring and artificially syntetized QSI compounds (85)(86). Screening for QSI in nature has identified many QSI compounds (87). These naturally occurring QSI compounds can be synthesized and their structure modified and used to inhibit QS *in vivo* in experimental animal infections (85). Since it has been shown, that bacteria used for experimental animal biofilm infections actually communicate *in vivo* (88) and also in e.g. CF patients with chronic *P. aeruginosa* lung infection QSI may be used to treat the infection (89). Interestingly, some macrolide antibiotics like azithromycin (90) but also other antibiotics like ceftazidime and ciprofloxacin (91) inhibit QS in *P. aeruginosa* at sub-MIC concentrations leading to inhibition of the virulence of these bacteria although they cannot inhibit their growth at obtainable concentrations *in vivo*. Controlled

clinical trials using azithromycin to treat the chronic *P. aeruginosa* lung infection in both CF children and adults have shown significant improvement of their lung function (92) (93) (94). Most CF patients with chronic *P. aeruginosa* lung infections are therefore now treated continously with azithromycin (95). An expected side-effect has, however, been development of resistance to macrolides in other pathogenic bacteria like *S. aureus* in CF patients (96). It would therefore be desireable to develop QSI without conventional growth-inhibiting or bacteriocidal activity (78). One example of such naturally occurring QSI is found in garlic extract, which *in vitro* and *in vivo* have been able to render otherwise resistant *P. aeruginosa* biofilms susceptible to antibiotic therapy and to PMN activity and the consequence is eradication of the biofilm both as regards antibiotic therapy and PMN activity which dominates the inflammatory response in CF patients (Fig. 7) (97). According to current knowledge, QSI resistance can only occur due to mutations, which renders the QS deficient bacteria unable to produce virulence factors (98) i.e. the bacteria become non-virulent similar to the result of QSI therapy. If this holds thrue, then resistance problems against conventional antibiotics as we face today will not be a clinical problem.

Foreign body infections constitute an steadily increasing medical problem and comprise e.g. intravenous catheters, intrauterine catheters, naso-laryngeal tubes, stents (Table 2, Fig. 1A), alloplastic materials, hydrocephalus shunts and artificial hearts (99) . If foreign bodies become colonized with biofilm-forming bacteria the result is most often chronic inflammation around the foreign body which either has to be replased or treated with sometimes life-long antibiotic suppressive therapy, although early therapy may sometimes lead to eradication of the condition. Antibiotic coated foreign bodies like e.g. catheters, vascular prostesis have been introduced to prevent biofilm formation and they are quite efficient (100) (101). It is, however, desirable to

develop other compounds due to the risk of development of bacterial resistance and QSIs are strong candidates. QSI have been shown synergistically to improve the weak effects of antibiotics and PMNs on biofilm growing bacteria *in vitro* and *in vivo* in animal experiments leading to elimination of biofilms (Fig. 7) (102). These results have lead to further development of QSI as pharmaceutical compounds for patients who are subject to implantation of foreign bodies.

9. **Prophylaxis and treatment of *P. aeruginosa* biofilms in CF lungs – perspectives for other biofilm infections?**

The currently used methods for preventing chronic *P. aeruginosa* biofilms in CF lungs are 1) prevention of cross-infection from other already chronically infected CF patients by isolation techniques and hygiejnic measures (103), 2) early aggressive eradication therapy of intermittent colonization by means of oral ciprofloxacin and nebulized colistin for 3 weeks or even better for 3 months or by using nebulized tobramycin as monotherapy (104), 3) daily nebulized DNase (Pulmozyme) (105). These 3 methods, which are combined in most CF centers, are successful and cost-efficient and has completely changed the epidemiology of chronic *P. aeruginosa* lung infection in CF patients from being very common in CF children to being predominantly a problem for adult patients and no problems of resistance to the antibiotics have been recorded (73, 106, 107) . Early aggressive eradication therapy has also been shown to superior in an animal model of P. aeruginosa infection in CF (108). Furthermore, although vaccines against *P. aeruginosa* have been developed and undergone clinical trials, they have not been further developed due to the success of the early, aggressive eradication therapy (109).

The recommended method for treatment of chronic P. aeruginosa biofilm

infection is chronic suppressive antibiotic therapy (2)(110) (Fig. 8) which is started when the chronic infection is diagnosed (continuous colonization at the monthly bacteriological examination for 6 months and/or increased level of antibodies against *P. aeruginosa* (111)(112) . The chronic suppressive therapy consists of daily nebulized colistin or tobramycin for the rest of the patient's life combined with either regular 2-week courses every 3 months of intravenous anti-pseudomonas antibiotics (combination therapy of 2 antibitics: tobramycin or colistin + ceftazidime, or piperacillin/tazobactam, or carbapenem, or aztreonam, or ciprofloxacin) or ad hoc intravenous therapy when clinical deteriorations occur (110). Additionally, DNase is inhaled every day to reduce the viscocity of the DNA-containing sputum (105), and daily oral azithromycin is given to the patients as mentioned above (95). The chronic suppressive therapy (maintenance therapy) has successfully been able to maintain the pulmonary function or slow the decline of the pulmonary function and prolong the life of the patients for many years (113). The side-effects of the maintenance therapy is high level of conventional resistance mechanisms in the persisting strains and high level of allergy to the beta-lactam antibiotics (114) (43). Similar principles, a) systemic or local prophylactic use of antibiotics to prevent biofilm formation, b) early aggressive eradication therapy to eradicate planktonic growth or early biofilm formation and c) chronic suppressive antibiotic therapy to maintain the function of a inserted foreign body are gradually being introduced in other area of biofilm infection (100) (8) (9). Possibly other approaches taken advantage of the use of QSI may further improve the management of biofilm infections.

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Conflict of interest statement

None to declare.

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Table 1. Some general features of biofilm infections in humans compared to acute planktonic infections and superficial colonozation/normal flora on skin and mucosal membranes. The bold fonts indicate biofilm specific features.

Features of biofilm infections	Necessary condition for biofilm infections	Sufficient condition for biofilm infecions	Also found in acute planktonic infections	Also found in colonization/ normal flora on skin and mucosal membranes
Aggregates of bacteria embedded in a self-produced polymer matrix	Yes	Yes	No	No/Yes
Tolerant to clinical relevant PK/PD dosing of antibiotics in spite of susceptibility of planktonic cells	Yes	Yes	No	No/Yes
Tolerant to both innate and adaptive immune reponse	Yes	Yes	No	No/Yes - unknown (s-IgA)
Inflammation	Yes	No	Yes	No
Biofilm-specific antigens	No and Yes - seldom – e.g. P.seudomonas aeruginosa alginate	No and Yes - seldom – e.g. Pseudomonas aeruginosa alginate	No	No
Antibody response	Yes - after some weeks	No	Yes - after some weeks	No
Chronic infections	Yes	Yes	No	No
Foreign body associated infections	No	Yes	No but yes the first day of infection	No
Located on surfaces	No	No	Yes	Yes
Localized infetion	Yes	No	Yes	Yes
Focus for spreading or local exacerbation	Yes	No	Yes	Yes

Table 2. Natural and pathogenic biofilms on human tissue and foreign bodies.

'Organ A' with normal flora	Connection via foreign bodies	'Organ B' without normal flora
Skin	→	blood, peritoneum, middle ear
Mouth	→	teeth
Pharynx	→	bronchi, lungs,
Duodenum	→	bile tract, pancreas
Urethra	→	urine bladder
Vagina	→	uterus
'Air in operation room'*	→	alloplastic, cerebrospinal shunt
No symptoms	→	pathologi

*most frequently coagulase negative staphylococci, which occur as biofilms on detached epidermal cells.

Antibiotic resistance of bacterial biofilms

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ABSTRACT

A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defence system. The persistence of, for example, staphylococcal infections related to foreign bodies is due to biofilm formation. Likewise, chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is caused by biofilm-growing mucoid strains. Characteristically, gradients of nutrients and oxygen exist from the top to the bottom of biofilms and these gradients are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells; it is these more or less dormant cells that are responsible for some of the tolerance to antibiotics. Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms. Conventional resistance mechanisms such as chromosomal β-lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms. Biofilms can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppressive therapy. A promising strategy may be the use of enzymes that can dissolve the biofilm matrix (e.g. DNase and alginate lyase) as well as quorum-sensing inhibitors that increase biofilm susceptibility to antibiotics.

1. Introduction

Biofilm-growing bacteria cause chronic infections [1] characterised by persistent inflammation and tissue damage [2]. Chronic infections, including foreign-body infections, are infections that (i) persist despite antibiotic therapy and the innate and adaptive immune and inflammatory responses of the host and (ii) in contrast to colonisation, are characterised by an immune response and persisting pathology (Table 1).

2. Occurrence and architecture of bacterial biofilms

Foreign-body infections are characterised by biofilm growth of bacteria on the outer and/or inner surface of the foreign body (Table 2). Biofilm growth also occurs on natural surfaces such as teeth [3], heart valves (endocarditis) [4], in the lungs of cystic fibrosis (CF) patients causing chronic bronchopneumonia [2], in the middle ear in patients with persistent otitis media [5], in chronic rhinosinusitis [6], in chronic osteomyelitis and prosthetic joint infections [7–9], in intravenous (i.v.) catheters and stents [10] and in chronic wounds [11,12] (Fig. 1). The microbes in biofilms are kept together by a self-produced biopolymer matrix. The matrix contains polysaccharides, proteins and DNA originating from the microbes, and the bacterial consortium can consist of one or more species living in sociomicrobiological way [1,2,14,15]. The matrix is important since it provides structural stability and protection to the biofilm. Development of bacterial biofilms over time has been intensively studied in vitro by confocal scanning laser microscopy employing green fluorescent protein (GFP)-tagged bacteria. This technique has been combined with

advanced in silico image analysis to produce three-dimensional images of the biofilm [16–18]. As an example, *Pseudomonas aeruginosa* produces a mature in vitro biofilm in 5–7 days (Fig. 2).

Development of an in vitro biofilm is initiated by planktonic (freely moving) bacteria that reversibly attach to a surface, which may be covered by a layer of, for example, proteins (a pellicle) [3,20]. At this stage, the bacteria are still susceptible to antibiotics and this is in accordance with the success of perioperative antibiotic prophylaxis, e.g. for alloplastic surgery. The next step is irreversible binding to the surface within the next few hours and multiplication of the bacteria, which form microcolonies on the surface and begin to produce a polymer matrix around the microcolonies [20]. The biofilm grows in thickness (up to 50 μm) and under in vitro conditions mushroom-like or tower-like structures are often observed in the mature biofilm. At that stage, the biofilm shows maximum tolerance (= resistance) to antibiotics. Subsequently, a stage follows where focal areas of the biofilm dissolve and the liberated bacterial cells can then spread to another location where new biofilms can be formed. This liberation process may be caused by bacteriophage activity within the biofilm [21]. The mature biofilm may contain water-filled channels and thereby resemble primitive, multicellular organisms. Motile bacteria can use type 1V pili to mount or climb a biofilm formed by other bacteria and colonise the top of the biofilm, resembling a hat [17]. Important properties of biofilm-growing bacteria are different from those of planktonic bacteria and this has significant diagnostic and therapeutic consequences. The bacteria appear different in biofilm infections since they

are located close to each other in aggregates surrounded by the self-produced matrix. In clinical specimens (biopsies, pus, sputum), biofilm can often be recognised by light microscopy, although precise identification of all the bacteria within a biofilm can only be done by DNA hybridisation techniques, and identification of the components of the biofilm matrix require specialised staining techniques [2]. Traditional sampling techniques may not be sufficient to culture biofilm-growing bacteria sticking to a surface unless the bacteria are released by ultrasonic pre-treatment [22]. Ordinary culture techniques, however, reveal only the properties of planktonically growing bacteria and, for example, antibiotic susceptibility testing therefore gives misleading results that do not reflect the increased resistance of the bacteria living in biofilms. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to biofilm-growing bacteria may be up to 100–1000-fold higher compared with planktonic bacteria [23–25]. Methods to test biofilm-growing bacteria have therefore been developed, but their clinical relevance with regard to prediction of clinically successful therapy awaits confirmation [24,26,27].

3. Stationary-phase physiology, low oxygen concentration and slow growth

Inspection of environmental as well as in vitro biofilms has revealed that the oxygen concentration may be high at the surface but low in the centre of the biofilm where anaerobic conditions may be present [28]. Likewise, growth, protein synthesis and metabolic activity is stratified in biofilms, i.e. a high level

of activity at the surface and a low level and slow or no growth in the centre, and this is one of the explanations for the reduced susceptibility of biofilms to antibiotics [29,30]. Very slow in situ growth rates of *P. aeruginosa* biofilms have been measured in the sputum of CF patients (average doubling time 2–3 h and the presence of a significant number of cells in stationary growth phase) [31]. Monotherapy with antibiotics such as β-lactams, which are only active against dividing *P. aeruginosa* cells, are therefore not very efficient at eradicating biofilm infections [23].

4. Mutators

The mutation frequency of biofilm-growing bacteria is significantly increased compared with planktonically growing isogenic bacteria [32] and there is increased horizontal gene transmission in biofilms [33]. These physiological conditions may explain why biofilm-growing bacteria easily become multidrug resistant by means of traditional resistance mechanisms against β-lactam antibiotics, aminoglycosides and fluoroquinolones, which are detected by routine susceptibility testing in the clinical microbiology laboratory where planktonic bacterial growth is investigated. Thus, bacterial cells in biofilms may simultaneously produce enzymes that degrade antibiotics, have antibiotic targets of low affinity and overexpress efflux pumps that have a broad range of substrates. Achievement of multiple mutations in a bacterial population size of 10^8–10^{10}/mL of sputum, as attained in infection of the CF lung [34], implies the presence of a hypermutable bacterial subpopulation, and the presence of

high percentages of hypermutable *P. aeruginosa* isolates associated with antibiotic resistance has actually been found in CF patients [35,36].

The hypermutable phenotype of CF *P. aeruginosa* isolates is due to alterations in genes of the DNA repair systems of either the mismatch repair system (MMR), which involves *mutS*, *mutL* and *uvrD*, or the DNA oxidative lesions repair system (GO), which involves *mutT*, *mutY* and *mutM* [37,38]. It has been shown that mutations in either of the two systems determine the emergence of antibiotic-resistant isolates, especially due to selection of isolates expressing multidrug efflux pumps [38,39]. Increased production of endogenous reactive oxygen species (ROS) and a deficient antioxidant system [40,41] determine an imbalance between oxidative burden and antioxidant defences leading to oxidative stress in biofilms. This oxidative stress is considered to cause enhanced mutability in biofilms [32,42]. Recent data suggest that microcolony structures, due to endogenous oxidative stress, are specific sites within biofilms for enhanced genetic adaptation and evolutionary change [42]. In addition, Boles and Singh [43] showed that the endogenous oxidative stress in biofilms promotes antibiotic resistance and that addition of antioxidants reduced the occurrence of diversity in biofilms. We have previously shown that oxidative stress is linked to the occurrence of hypermutable *P. aeruginosa* strains in CF patients [36].

In addition to their endogenous oxidative stress, biofilm-growing bacteria in the CF airways are exposed to ROS from activated polymorphonuclear leukocytes (PMNs) [36]. We have recently shown that the hypoxic

environment in CF sputum is due to the consumption of oxygen by PMNs which liberate ROS that can react with the biofilm-embedded bacterial cells [44], thus creating an unique environment with low oxygen tension filled with ROS.

The hypermutability of bacteria in biofilms promotes the emergence of mutations conferring antibiotic resistance, which will be selected for by the repeated antibiotic courses administered in order to maintain the lung function of CF patients. Development of resistance to all classes of antibiotics during chronic lung infection in CF has been documented [45]. Resistance to β-lactam antibiotics occurs due to mutations in the regulatory genes of β-lactamase production leading to the occurrence of isolates with stable or partially stable derepressed production of AmpC β-lactamase [46]. Resistance to ciprofloxacin of CF *P. aeruginosa* isolates was shown to be mediated by mutations in *gyrA* and alterations in two efflux systems (MexCD-OprJ and MexEF-OprN), and resistance to tobramycin was due to overexpression of the MexXY-OprM multidrug efflux pump [47,48]. Resistance to colistin was shown to occur due to mutations in the *pmr* system involved in the lipopolysaccharide (LPS) structure [49].

5. Chromosomal β-lactamase and biofilm matrix components

Overproduction of chromosomally encoded AmpC cephalosporinase is considered the main mechanism of resistance of CF *P. aeruginosa* isolates to β-lactam antibiotics [50]. The most common β-lactamase production

phenotype in CF isolates is the partially derepressed phenotype with high basal levels of β-lactamase that can be further induced to higher levels in the presence of β-lactam antibiotics [46]. The role of this β-lactamase phenotype is especially important for resistance to β-lactam antibiotics acting as strong inducers (carbapenems such as imipenem). However, not all β-lactams are strong inducers and overexpression of the MexAB-OprM efflux pump may, together with β-lactamases, play an important role in resistance to poor inducers (e.g. piperacillin). Totally derepressed β-lactamase production is encountered in 2.5% of clinical CF isolates [46] and is responsible for resistance both to poor- and strong-inducer β-lactam antibiotics, independent of the presence of efflux pump overexpression [51,52]. We have found an insertion sequence (IS*1669*) inactivating the *ampD* gene in several resistant clinical *P. aeruginosa* isolates with constitutive high expression of chromosomal β-lactamase [52].

The diffusion barrier in biofilms [53] plays a role in biofilm resistance of *P. aeruginosa* that overproduce β-lactamase owing to the presence in the biofilm matrix of β-lactamases that will hydrolyse the β-lactam antibiotics before reaching the bacterial cells [54,55]. Giwercman et al. [56] showed that imipenem and piperacillin were able to induce β-lactamase production in *P. aeruginosa* biofilms. Nichols et al. [57] predicted from mathematical models that the biofilm would not afford protection against diffusion of β-lactam antibiotics into the bacteria embedded in the biofilm as long as the level of chromosomal β-lactamase was low. However, bacteria expressing a high

level of chromosomal β-lactamase growing in biofilms would be exposed to a reduced concentration of β-lactam antibiotics owing to accumulation of the enzyme in the polysaccharide matrix. The extracellular β-lactamase would inactivate the antibiotic as it penetrates, thereby protecting the deeper-lying cells.

The source of β-lactamase in biofilms has been considered to be from a layer of lysed bacteria owing to exposure to an antibiotic, with release of defensive enzymes into the extracellular space. We have shown that the source of β-lactamase in biofilm may also be the membrane vesicles containing β-lactamase liberated by resistant *P. aeruginosa* bacteria [55,58] and we have shown that a high level of free chromosomal β-lactamase is present in CF sputum [58]. We have also shown that strong inducers such as imipenem will induce the β-lactamase through all the bacterial layers, whilst poorer inducers such as ceftazidime will influence just the superficial layers of the biofilm, probably due to inactivation of the antibiotic by β-lactamase [58–60] (Fig. 3).

The protective role played by β-lactamases in impairing the penetration of β-lactams in the biofilm can be seen in Fig. 3A. Treatment with ceftazidime of a biofilm formed by a *P. aeruginosa* CF strain with stable derepressed levels of β-lactamase due to an insertion sequence in *ampD* (*P. aeruginosa ampD*$^{-}$) killed very few bacterial cells (dead bacteria in red) (Fig. 3B) in contrast to the complemented strain with a low level of β-lactamase (*P. aeruginosa ampD*$^{+}$) (Fig. 3C). However, addition of aztreonam improved the efficacy of ceftazidime treatment of the biofilm (Fig. 3D), probably because aztreonam

acts as a β-lactamase inhibitor [58]. In addition, meropenem, a β-lactamase-stable β-lactam, showed good in vitro efficacy in the treatment of *P. aeruginosa* biofilms [24,60]. Treatment with ceftazidime of a biofilm formed by the same strain expressing basal levels of β-lactamase due to complementation with the wild-type *ampD* (*P. aeruginosa ampD*$^{+}$) led to eradication of the biofilm (Fig. 3C).

The matrix of the biofilm may also be part of the resistance mechanism to antibiotics since, for example, sub-MIC concentrations of β-lactam antibiotics induce increased alginate synthesis in *P. aeruginosa* biofilms (Fig. 4) [62,63] and also enhance the biofilm matrix of some slime-producing coagulase-negative staphylococci [64,65]. Originally, it was thought that tolerance of biofilms to aminoglycosides was the result of transport limitation due to the binding of these positively charged antibiotics to the negatively charged exopolysaccharide matrix, but the repeated dosing of antibiotics during therapy probably leads to saturation of the binding sites [66–68]. As discussed previously, oxygen limitation and the metabolic rate are probably more important factors contributing to the tolerance of biofilms to aminoglycosides and ciprofloxacin [69]. However, in the respiratory zone of the CF lung with poor access to aminoglycoside aerosols, where the antibiotic concentration is low, delayed penetration of aminoglycosides through thick biofilms may play a role in the tolerance of biofilms to these aminoglycosides [2]. Recently, it has been shown that administration of DNase and alginate lyase enhanced the activity of tobramycin in biofilms by dissolving the biofilm matrix [70].

6. Tolerance, adaptive resistance and efflux pumps

Colistin is only antimicrobial active against the non-dividing central part of *P. aeruginosa* biofilms in vitro (Fig. 5A), whereas the superficial, metabolically active part of the biofilm becomes tolerant due to upregulation of the PmrA-PmrB two-component regulatory system involved in adaptive resistance to cationic peptides leading to addition of aminoarabinose to lipid A of LPS [24,71,72]. Since the metabolically active surface layer of the biofilm is susceptible to ciprofloxacin (Fig. 5B), in contrast to the dormant central part of the biofilm, combination therapy with ciprofloxacin and colistin was able to kill all cells in the biofilm in vitro (Fig. 5C) [71]. Clinical efficacy has been demonstrated with this combination therapy for the early eradication treatment of *P. aeruginosa* in CF patients [73].

Tolerance of biofilms to tobramycin is also mediated by low metabolic activity, but the high cell density that results in accumulation of extracellular signalling molecules is probably important, as it has been shown that tolerance to tobramycin of *P. aeruginosa* strain PAO1 biofilm is quorum-sensing-mediated (Fig. 6) [74] (see below). In addition, a non-specific mechanism for the tolerance of the metabolically active cells to colistin was shown to be upregulation of the MexAB-OprM efflux pump [71]. Furthermore, increased efflux pump activity due to mutations has been shown to be a major resistance mechanism against aminoglycoside antibiotics and fluoroquinolones in *P. aeruginosa* from CF patients [47,48].

7. High cell density and quorum sensing (QS)

Bacteria communicate by means of synthesising and reacting on signal molecules [75–78]. The term QS indicates that this system allows bacteria to sense when a critical number (concentration) of bacteria are present in a limited space in the environment and respond by activating certain genes that then produce, for example, virulence factors such as enzymes or toxins. The QS molecules are small peptides in many Gram-positive bacteria, whereas the most well described QS molecules in Gram-negative bacteria are *N*-acyl-L-homoserine lactones [78]. For *P. aeruginosa*, QS regulates the production of virulence factors such as extracellular enzymes and cellular lysins (e.g. rhamnolipid), which are important for the pathogenesis of infections where it functions as a protective shield against phagocytes [79–81]. QS may also influence the development of the biofilm [82] and QS has been shown to determine the tolerance of *P. aeruginosa* biofilms to antibiotic therapy and to the innate inflammatory response dominated by PMNs [74]. The connection between QS and biofilms has been named sociomicrobiology [83].

8. Quorum-sensing inhibitors (QSIs)

Much of our knowledge about QS originates from experiments with QS knock-out mutants and from the use of naturally occurring and artificially synthesised QSI compounds [84,85]. Screening for QSIs in nature has identified many QSI compounds [86]. These naturally occurring QSI compounds can be synthesised and their structure modified and used to inhibit QS in vivo in experimental animal infections [84]. Since it has been shown that bacteria

used for experimental animal biofilm infections actually communicate in vivo [87] and also in, for example, CF patients with chronic *P. aeruginosa* lung infection, QSIs may be used to treat these infections [88]. Interestingly, some macrolide antibiotics such as azithromycin [89], but also other antibiotics such as ceftazidime and ciprofloxacin [90], inhibit QS in *P. aeruginosa* at sub-MIC concentrations, leading to inhibition of the virulence of these bacteria even though they cannot inhibit their growth at concentrations obtainable in vivo. Controlled clinical trials using azithromycin to treat chronic *P. aeruginosa* lung infection both in CF children and adults have shown significant improvement of their lung function [91–93]. Most CF patients with chronic *P. aeruginosa* lung infections are therefore now treated continuously with azithromycin [94]. An expected side effect, however, has been the development of resistance to macrolides in other pathogenic bacteria such as *Staphylococcus aureus* in CF patients [95]. It would therefore be desirable to develop QSIs without conventional growth-inhibiting or bactericidal activity [78]. One example of such a naturally occurring QSI is found in garlic extract, which in vitro and in vivo has been able to render otherwise resistant *P. aeruginosa* biofilms susceptible to antibiotic therapy and to PMN activity and the consequence is eradication of the biofilm both with regard to antibiotic therapy and PMN activity which dominates the inflammatory response in CF patients (Fig. 7) [96]. According to current knowledge, QSI resistance can only occur due to mutations, which render the QS-deficient bacteria unable to produce virulence factors [97], i.e. the bacteria become non-virulent, similar to the result of QSI therapy. If this holds true, then resistance problems against conventional antibiotics as we face today will not be a clinical problem.

Foreign-body infections constitute a steadily increasing medical problem and comprise, for example, infections of i.v. catheters, intrauterine catheters, nasolaryngeal tubes, stents (Table 2; Fig. 1A), alloplastic materials, hydrocephalus shunts and artificial hearts [98]. If foreign bodies become colonised with biofilm-forming bacteria the result is most often chronic inflammation around the foreign body that either has to be replaced or treated with sometimes life-long antibiotic suppressive therapy, although early therapy may sometimes lead to eradication of the condition. Antibiotic-coated foreign bodies, e.g. catheters and vascular prostheses, have been introduced to prevent biofilm formation and they are quite efficient [99,100]. However, it is desirable to develop other compounds owing to the risk of development of bacterial resistance, and QSIs are strong candidates. QSIs have been shown to improve synergistically the weak effects of antibiotics and PMNs on biofilm-growing bacteria in vitro and in vivo in animal experiments, leading to elimination of biofilms (Fig. 7) [79]. These results have led to further development of QSIs as pharmaceutical compounds for patients who are subject to implantation of foreign bodies.

9. Prophylaxis and treatment of *Pseudomonas aeruginosa* biofilms in cystic fibrosis lungs: perspectives for other biofilm infections?

The currently used methods for preventing chronic *P. aeruginosa* biofilms in CF lungs are (i) prevention of cross-infection from other already chronically

infected CF patients by isolation techniques and hygienic measures [101], (ii) early aggressive eradication therapy of intermittent colonisation by means of oral ciprofloxacin and nebulised colistin for 3 weeks or, even better, for 3 months or by using nebulised tobramycin as monotherapy [102] and (iii) daily nebulised DNase (Pulmozyme®) [103]. These three methods, which are combined in most CF centres, are successful and cost efficient and have completely changed the epidemiology of chronic *P. aeruginosa* lung infection in CF patients from being very common in CF children to being predominantly a problem for adult patients, and no problems of resistance to the antibiotics have been recorded [73,104,105]. Early aggressive eradication therapy has also been shown to superior in an animal model of *P. aeruginosa* infection in CF [106]. Furthermore, although vaccines against *P. aeruginosa* have been developed and have undergone clinical trials, they have not been further developed owing to the success of early aggressive eradication therapy [107].

The recommended method for treatment of chronic *P. aeruginosa* biofilm infection is chronic suppressive antibiotic therapy [2,108] (Fig. 8), which is started when the chronic infection is diagnosed (continuous colonisation at the monthly bacteriological examination for 6 months and/or increased level of antibodies against *P. aeruginosa*) [109,110]. Chronic suppressive therapy consists of daily nebulised colistin or tobramycin for the rest of the patient's life combined with either regular 2-week courses every 3 months of i.v. antipseudomonal antibiotics (combination therapy of two antibiotics: tobramycin or colistin + ceftazidime, or piperacillin/tazobactam, or carbapenem, or aztreonam, or ciprofloxacin) or ad hoc i.v. therapy when

clinical deterioration occurs [108]. Additionally, DNase is inhaled every day to reduce the viscosity of the DNA-containing sputum [103], and daily oral azithromycin is given to patients as mentioned above [94]. Chronic suppressive therapy (maintenance therapy) has successfully been able to maintain pulmonary function or slow the decline in pulmonary function and prolong the life of patients for many years [111]. The side effects of maintenance therapy is a high level of conventional resistance mechanisms in the persisting strains and a high level of allergy to β-lactam antibiotics [45,112]. Similar principles, e.g. (a) systemic or local prophylactic use of antibiotics to prevent biofilm formation, (b) early aggressive eradication therapy to eradicate planktonic growth or early biofilm formation and (c) chronic suppressive antibiotic therapy to maintain the function of an inserted foreign body, are gradually being introduced in other areas of biofilm infection [8,9,99]. Possibly other approaches taking advantage of the use of QSIs may further improve the management of biofilm infections.

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Fig. 1. (A) Example of a biofilm on a biliary stent, which became the focus of repeated incidents of sepsis that were ultimately lethal. DNA typing (pulsed-field gel electrophoresis) showed that it was the same clonal type of *Escherichia coli* in the biofilm and in the blood. Despite relevant antibiotic therapy, it was not possible to eradicate the biliary focus on the stent and the second incident of sepsis was lethal. Microphotos: Gram and methylene blue staining, magnification ×100 and ×1000. Reproduced with permission from [13]. (B) Chronically infected wound with microcolonies of *Pseudomonas aeruginosa* surrounded but not penetrated by polymorphonuclear leukocytes (PMNs). Bacteria are identified using fluorescence in situ hybridisation (FISH) using a fluorescein-labelled peptide nucleic acid specific for *P. aeruginosa*. PMNs were stained by 4',6-diamidino-2-phenylindole (DAPI). Reproduced from [11] with permission.

Fig. 2. Time course of formation of a *Pseudomonas aeruginosa* biofilm based on in vitro experiments with green fluorescent protein-tagged *P. aeruginosa*, grown in a flow cell and examined using confocal scanning laser microscopy. QS (quorum sensing) indicates cell-to-cell communication, and EPS is the hydrated extracellular biofilm matrix. The thickness of the biofilm varies between 25 μm and 100 μm and is determined by the balance between growth and liberation of bacteria. Reproduced from [19] with permission.

Fig. 3. (A) Induction of β-lactamase in *Pseudomonas aeruginosa* biofilm. *Pseudomonas aeruginosa* PAO1 expressing green fluorescent protein (GFP) when the promoter of the AmpC β-lactamase is induced (P*ampC-gfp*): 6-day-

old biofilm exposed to 100 μg/mL ceftazidime for 4 h. Detection level of the monitor, 10 μg/mL ceftazidime. Reproduced from [61] with permission. (B) Treatment of *P. aeruginosa* biofilm with a β-lactam. *Pseudomonas aeruginosa ampD*$^{-}$ (levels of AmpC β-lactamase: basal 1050 mU; induced 4255 mU) expressing GFP as a tag. Seven-day-old biofilm treated with 10× minimal inhibitory concentration (MIC) of ceftazidime. Propidium iodide (PI) was added after Day 6 to monitor continuously the killing of the biofilm by ceftazidime. Reproduced from [61] with permission. (C) Treatment of *P. aeruginosa* biofilm with a β-lactam. *Pseudomonas aeruginosa ampD*$^{+}$ (levels of AmpC β-lactamase: basal 3 mU; induced 175 mU) expressing GFP as a tag. Seven-day-old biofilm treated with 10× MIC of ceftazidime. PI was added after Day 6 to monitor continuously the killing of the biofilm by ceftazidime. Reproduced from [61] with permission. (D) Treatment of *P. aeruginosa* biofilm with a combination of ceftazidime and aztreonam. *Pseudomonas aeruginosa ampD*$^{-}$ (levels of AmpC β-lactamase: basal 1050 mU; induced 4255 mU) expressing GFP as a tag. Seven-day-old biofilm treated with a combination of ceftazidime and aztreonam (10× MICs). PI was added after Day 6 to monitor continuously the killing of the biofilm by ceftazidime. Reproduced from [61] with permission.

Fig. 4. Induction of alginate in *Pseudomonas aeruginosa* biofilms treated with sub-minimal inhibitory concentrations (MICs) of imipenem [62]. (A) *Pseudomonas aeruginosa* PAO1 not exposed to antibiotics; (B) PDO300 (a PAO1 derivative constitutively expressing alginate) not exposed to antibiotics; (C) PAO1 exposed to imipenem for 18 h; and (D) PAO1 biofilm exposed to imipenem for 37 h. Alginate is stained green by concanavalin A-conjugated

fluorescein isothiocyanate (conA-FITC). Reproduced from [62] with permission.

Fig. 5. (A) Treatment of *Pseudomonas aeruginosa* biofilm with colistin. *Pseudomonas aeruginosa* PAO1 expressing green fluorescent protein (GFP) as a tag was grown as a biofilm in a flow chamber for 4 days. Propidium iodide (PI) was added after Day 4 to monitor continuously the killing of the biofilm by colistin. Image shows the biofilm after 2 days of treatment with 25 μg/mL colistin. Reproduced from [71] with permission. (B) Treatment of *P. aeruginosa* biofilm with ciprofloxacin. *Pseudomonas aeruginosa* PAO1 expressing GFP as a tag was grown as a biofilm in a flow chamber for 4 days and was treated for 2 days with 10 μg/mL ciprofloxacin. PI was added after Day 4 to monitor continuously the killing of the biofilm by ciprofloxacin. Red staining shows that ciprofloxacin kills the bacteria located at the surface of the biofilm. Reproduced from [71] with permission. (C) Treatment of *P. aeruginosa* biofilm with a combination of ciprofloxacin and colistin. *Pseudomonas aeruginosa* PAO1 expressing GFP as a tag was grown as a biofilm in a flow chamber for 4 days. PI was added after Day 4 to monitor continuously the killing of the biofilm by ciprofloxacin and colistin. The image shows the biofilm after 2 days of treatment with 10 μg/mL ciprofloxacin and 25 μg/mL colistin. Reproduced from [71] with permission.

Fig. 6. Treatment of *Pseudomonas aeruginosa* biofilm with tobramycin. Wild-type PAO1 and ΔlasRrhlR mutant, both expressing green fluorescent protein as a tag, were grown as biofilms in flow chambers for 3 days. On Day 3,

tobramycin 10 μg/mL and 20 μg/mL was added. Propidium iodide (PI) was added after Day 3 to monitor continuously the killing of the biofilm by tobramycin. Image shows the biofilm after 48 h of treatment: (a) untreated wild-type; (b) 10 μg/mL tobramycin-treated wild-type; (c) 20 μg/mL tobramycin-treated wild-type; (d) untreated ΔlasRrhlR mutant; (e) 10 μg/mL tobramycin-treated ΔlasRrhlR mutant; and (f) 20 μg/mL tobramycin-treated ΔlasRrhlR mutant. Reproduced with permission from [74].

Fig. 7. Four-day-old biofilm formed by green fluorescent protein-tagged *Pseudomonas aeruginosa* cultured in (A,B) the absence and (C,D) the presence of 2% garlic extract. Biofilms in B and D were treated on Day 3 with 340 μg/mL tobramycin for 24 h. Biofilms were then stained with LIVE/DEAD BacLight™ Bacterial Viability Kit, where dead bacteria are red and living bacteria are green. It is seen that in the presence of garlic extract + tobramycin can kill the bacteria in the biofilm whereas they survive if tobramycin or garlic is used alone. Reproduced from [74] with permission.

Fig. 8. Gram stain (×1000) of an explanted lung with a biofilm of *Pseudomonas aeruginosa* surrounded by numerous polymorphonuclear leukocytes. The patient is a 41-year-old cystic fibrosis male who has suffered from chronic mucoid *P. aeruginosa* lung infection for 28 years and has been treated with 114 courses of 2-week antipseudomonal antibiotic treatment (chronic suppressive maintenance therapy, total: 1 kg tobramycin, 10 kg β-lactam antibiotics and 1 kg colistin). He had developed 46 precipitating antibodies against *P. aeruginosa* (normal, 0–1).

Table 1

Some general features of biofilm infections in humans compared with acute planktonic infections and superficial colonisation/normal flora on skin and mucosal membranes. Bold indicates biofilm-specific features

Features of biofilm infections	Necessary condition for biofilm infection	Sufficient condition for biofilm infection	Also found in acute planktonic infection	Also found in colonisation/normal flora on skin and mucosal membranes
Aggregates of bacteria embedded in a self-produced polymer matrix	Yes	Yes	No	No/Yes
Tolerant to clinically relevant PK/PD dosing of antibiotics despite susceptibility of planktonic cells	Yes	Yes	No	No/Yes

Tolerant both to innate and adaptive immune responses	Yes	Yes	No	No/Yes—unknown (sIgA)
Inflammation	Yes	No	Yes	No
Biofilm-specific antigens	No and yes–seldom, e.g. *Pseudomonas aeruginosa* alginate	No and yes—seldom, e.g. *P. aeruginosa* alginate	No	No
Antibody response	Yes—after some weeks	No	Yes—after some weeks	No
Chronic infections	Yes	Yes	No	No
Foreign-body-associated infections	No	Yes	No, but yes the first day of infection	No
Located on surfaces	No	No	Yes	Yes
Localised infection	Yes	No	Yes	Yes
Focus for spreading or local exacerbation	Yes	No	Yes	Yes

PK/PD, pharmacokinetic/pharmacodynamic; sIgA, serum immunoglobulin A.

. 1

Table 2

Natural and pathogenic biofilms on human tissue and foreign bodies

'Organ A' with normal flora	Connection via foreign bodies	'Organ B' without normal flora
Skin	→	blood, peritoneum, middle ear
Mouth	→	teeth
Pharynx	→	bronchi, lungs
Duodenum	→	bile tract, pancreas
Urethra	→	urine, bladder
Vagina	→	uterus
'Air in operation room' [a]	→	alloplastic, cerebrospinal shunt
No symptoms	→	pathology

[a] Most frequently, coagulase-negative staphylococci, which occur as biofilms on detached epidermal cells.

Fig. 1A.

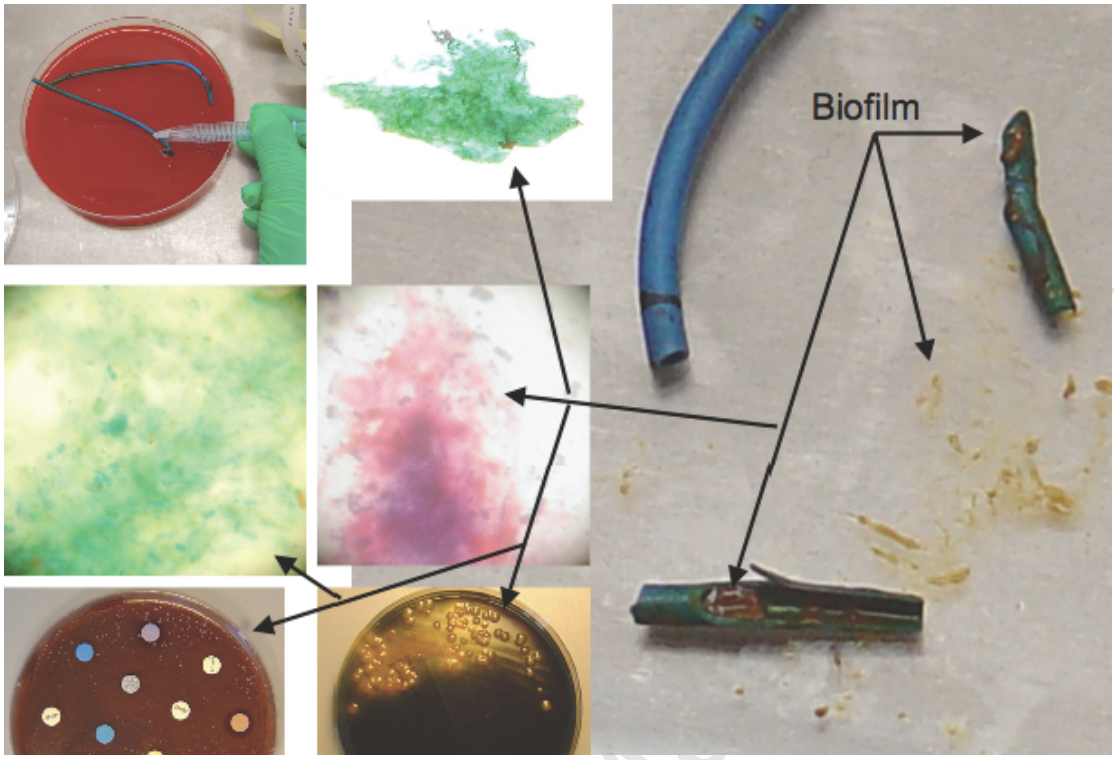

Fig. 1B.

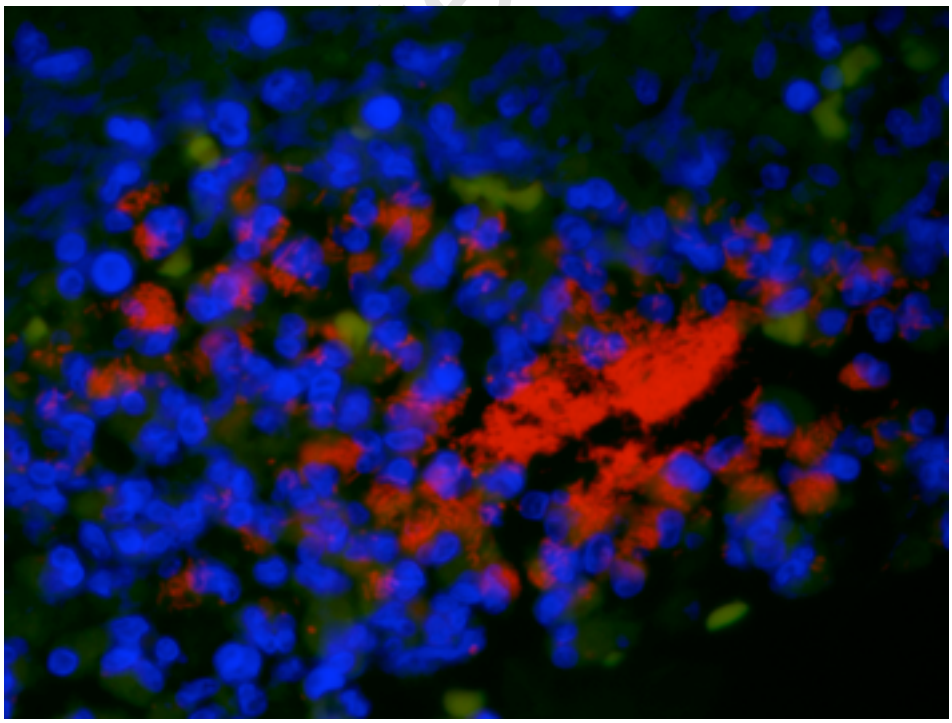

Figure 1. *A*. Example of a biofilm on a biliary stent which became focus of repeated incidents of sepsis which ultimately was lethal. DNA typning (pulsed field gel electrophorese) showed that it was the same cloneal type of *E. coli* in the biofilm and in the blood. In spite of relevant antibiotic therapy it was not possible to eradicate the biliary focus on the stent and the second incident of sepsis was lethal. Microphotos: Gram- and methylene blue staining, magnification x 100 and x 1000. Reproduced with permission from Høiby et al. Ugeskrift for Læger 169:4163-4166;, 2007. *B* Chronic infected wound with microcolonies of *P. aeruginosa* surrounded but not penetrated by polymorphonuclear leukocytes. The bacteria are identified by use of fluorescence in situ hybridization (FISH) using a fluorescein-labelled (FITC) peptide nucleic acid (PNA) specific for *P. aeruginosa.* The polymorphonuclear leukocytes were stained by DAPI. Reproduced from (11) with permission.

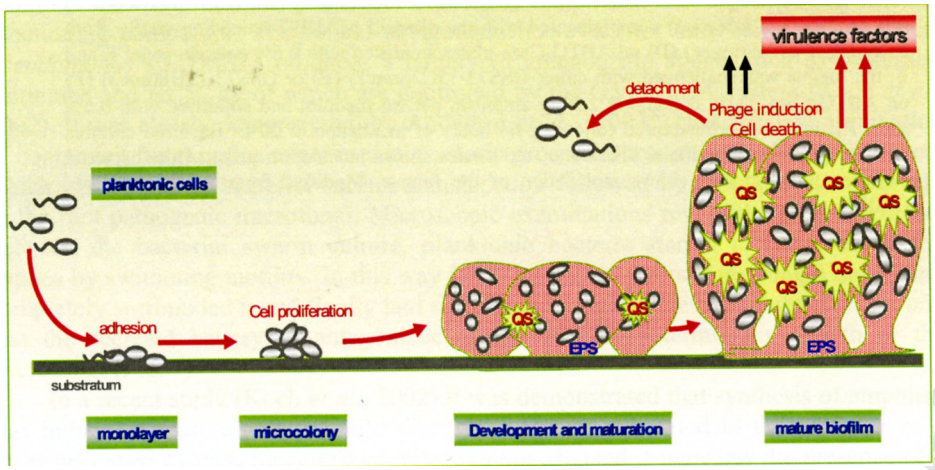

Figure 2. Time-course of formation of a *P. aeruginosa* biofilm based on *in vitro* experiments with *green fluorescent protein*-tagged *P. aeruginosa*, which grow in a *flow-cell* and is examined with a confocal scanning laser microscop. QS indicates cell-to-cell communication and EPS is hydrated extraclellular biofilm matrix. The thickness of the biofilm varies between 25 – 100 µm and is determed by the balance between growth and libetation of the bacteria. Reproduced from (115) with permission.

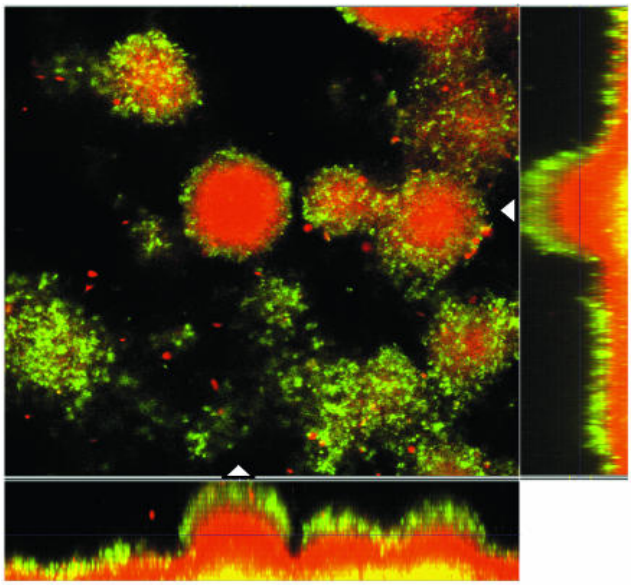

Figure 3A. Induction of beta-lactamase in *P. aeruginosa* biofilm. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) when the promoter of the AmpC beta-lactamase is induced (P*ampC-gfp*): 6 days old biofilm exposed to 100 µg/ml ceftazidime for 4 h. Detection level of the monitor: 10 µg/ml ceftazidime. Reproduced from (116) with permission.

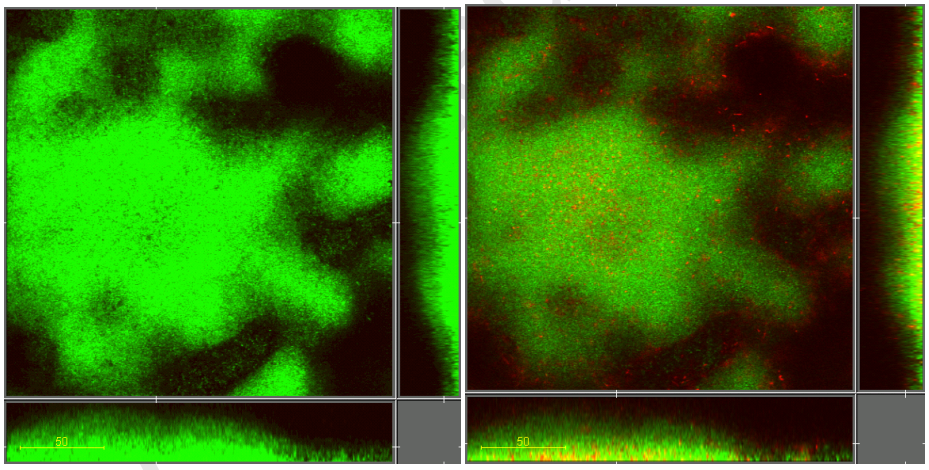

Figure 3B. Treatment of *P. aeruginosa* biofilm with beta-lactams. *P. aeruginosa ampD*$^{-}$ (levels AmpC beta-lactamase (mU) :1050 basal, 4255 induced) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with 10 times MIC of ceftazidime. Propidium iodide was added after day 6 to continuosly monitor the killing of the biofilm by ceftazidime. Reproduced from (116) with permission.

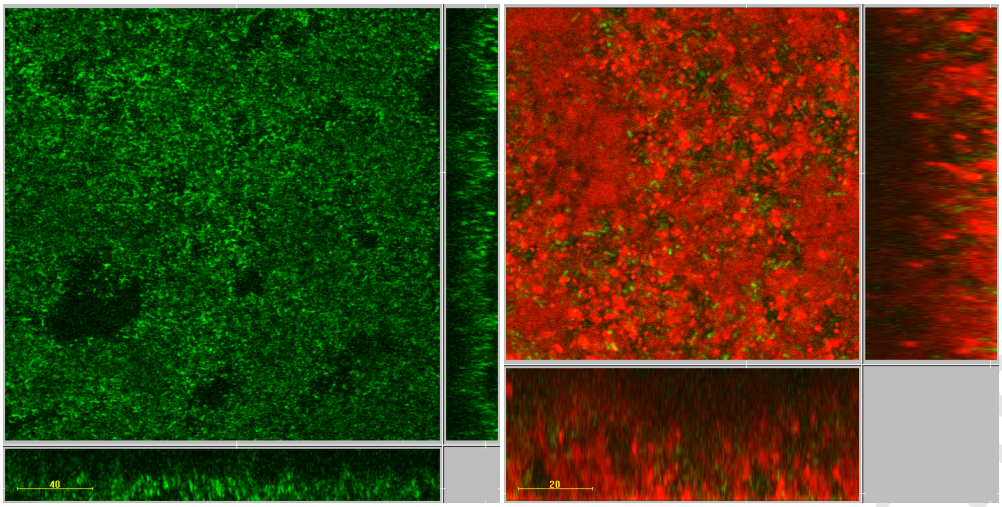

Figure 3C. Treatment of *P. aeruginosa* biofilm with beta-lactams. *P. aeruginosa ampD*+ (levels AmpC beta-lactamase (mU): basal 3, induced 175) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with 10 times MIC of ceftazidime. Propidium iodide was added after day 6 to continuosly monitor the killing of the biofilm by ceftazidime. Reroduced from (116) with permission.

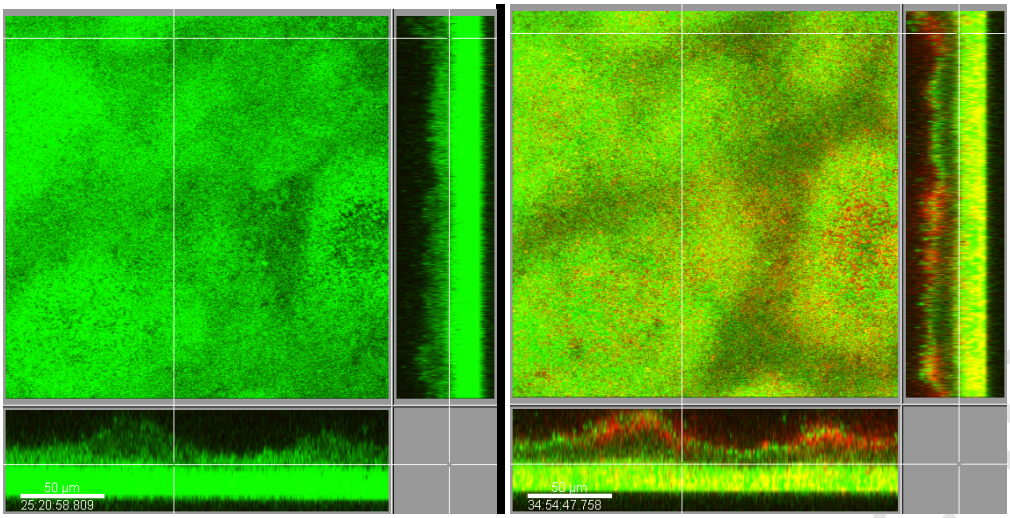

Figure 3D. Treatment of P. aeruginosa biofilms with a combination of ceftazidime and aztreonam. *P. aeruginosa ampD*$^{-}$ (levels AmpC beta-lactamase (mU) :1050 basal, 4255 induced) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with a combination of ceftazidime and aztreonam (10 times MICs). Propidium iodide was added after day 6 to continuosly monitor the killing of the biofilm by ceftazidime. Reproduced from (116) with permission.

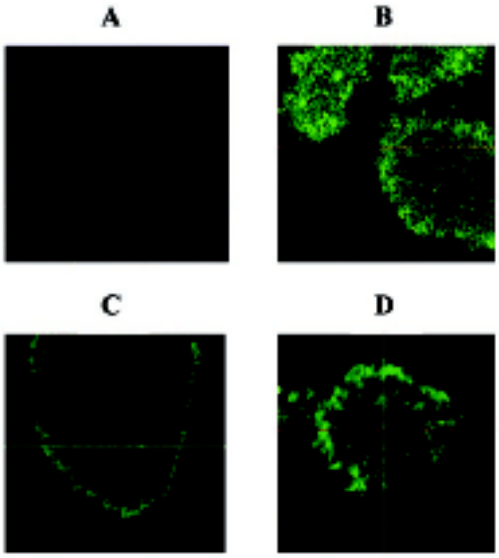

Figure 4 Induction of alginate in *P. aeruginosa* biofilms treated with sub-MIC concentrations of imipenem (62) A. *P. aeruginosa* PAO1 not exposed to antibiotics; B. PDO300 (a PAO1 derivative constitutively expressing alginate) not exposed to antibiotics; C. PAO1 exposed to imipenem for 18 hours; D. PAO1 biofilm exposed to imipenem for 37 hours. Alginate is stained green by conA-FITC. Reproduced from (117) with permission.

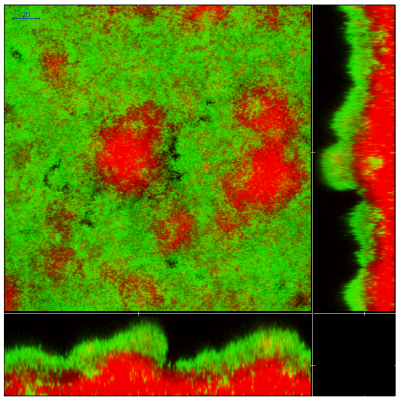

Figure 5A. Treatment of *P. aeruginosa* biofilms with colistin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag were grown as biofilms in flow-chambers for 4 days. Propidium iodide was added after day 4 to continuosly monitor the killing of the biofilm by colistin. The picture shows the biofilm after 2 days of treatment with colistin 25 µg/ml. Reproduced from(71) with permission.

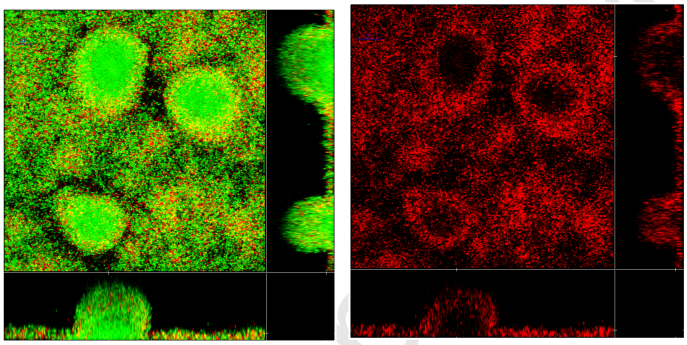

Figure 5 B. Treatment of *P. aeruginosa* biofilms with ciprofloxacin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag was grown as biofilm in a flow-chamber for 4 days and was treated for 2 days with ciprofloxacin 10 µg/ml (A). Propidium iodide was added after day 4 to continuosly monitor the killing of the biofilm by ciprofloxacin. Red staining shows that ciprofloxacin kills the bacteria located at the surface of the biofilm. Reproduced from (71)with permission.

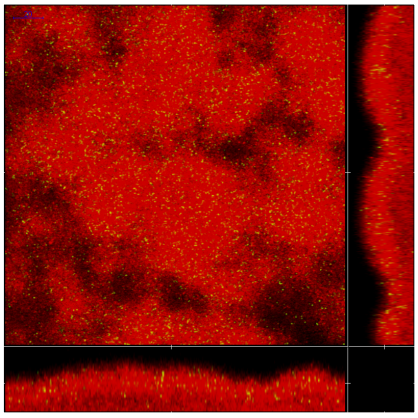

Figure 5C Treatment of *P. aeruginosa* biofilm with a combination of ciprofloxacin and colistin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag was grown as biofilm in flow-chambers for 4 days. Propidium iodide was added after day 4 to continuosly monitor the killing of the biofilm by ciprofloxacin and colistin. The present picture shows the biofilm after 2 days of treatment with 10 µg/ml ciprofloxacin and 25 µg/ml colistin. Reproduced from (71) with permission.

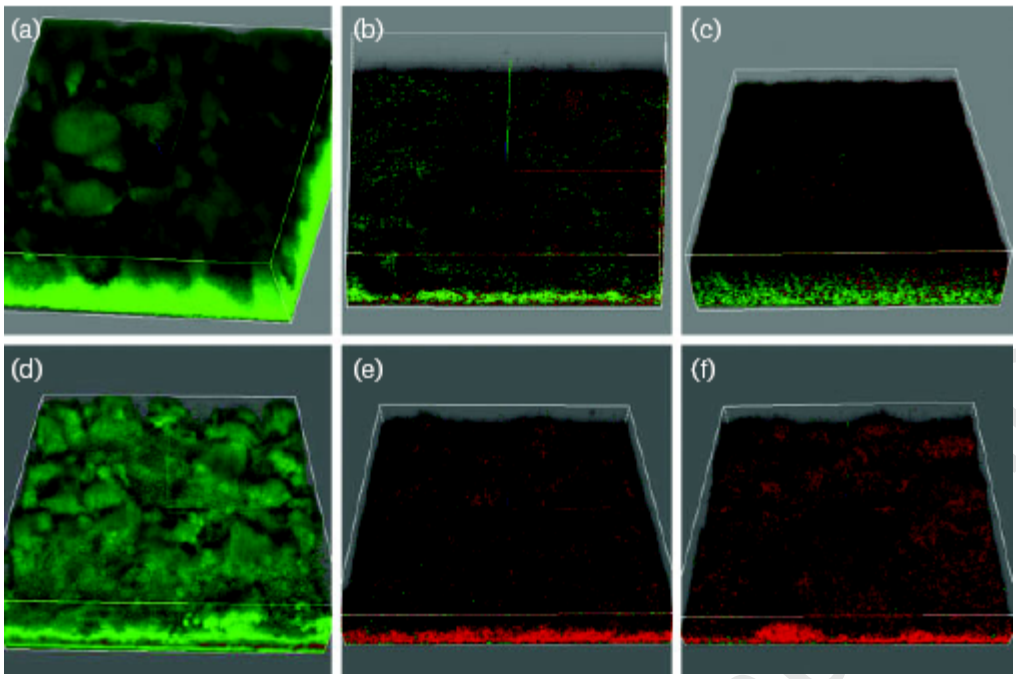

Figure 6. Treatment of P. *aeruginosa* biofilm with tobramycin. Wild-type PAO1 and ΔlasRrhlR mutant, both expressing green-fluorescent protein (gfp) as a tag were grown as biofilms in flow-chambers for 3 days. On day 3 tobramycin 10 µg/ml and 20 µg/ml were added. Propidium iodide was added after day 3 to continuosly monitor the killing of the biofilm by tobramycin. The present pictures show the biofilm after 48 hours of treatment. (a) untreated wild-type, (b) 10 µg/ml wild-type, (c) 20 µg/ml wild-type, (d) untreated *ΔlasRrhlR* mutant, (e) 10 µg/ml *ΔlasRrhlR* mutant, (f) 20 µg/ml *ΔlasRrhlR* mutant. Reproduced with permission from (74) .

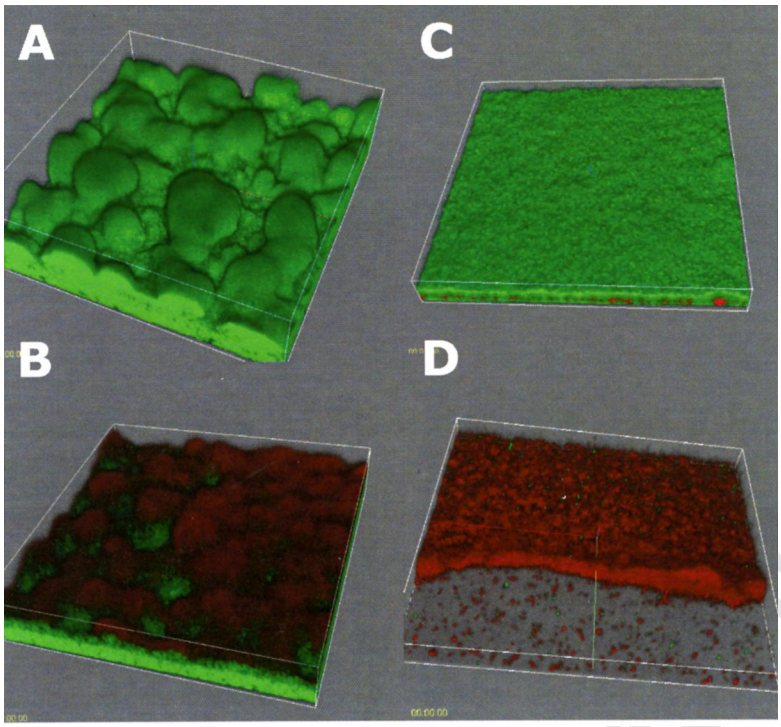

Fig. 7. A 4 day old biofilm formed by *green fluorescent protein*-tagged *P. aeruginosa* cultured in absense (A and B) or presence (C and D) of 2% garlic extract. The biofilms B and D were treated on day 3 with 340 µg/ml tobramycin for 24 h. The biofilms were then stained with LIVE/DEAD *Bac*Light bacterial Viability Kit, where dead bacteria are red and living bacteria are green. It is seen that in the presence of garlic extract + tobramycin can kill the bacteria in the biofilm whereas they survive if tobramycin or garlic is used alone. Reproduced from (74) with persission.

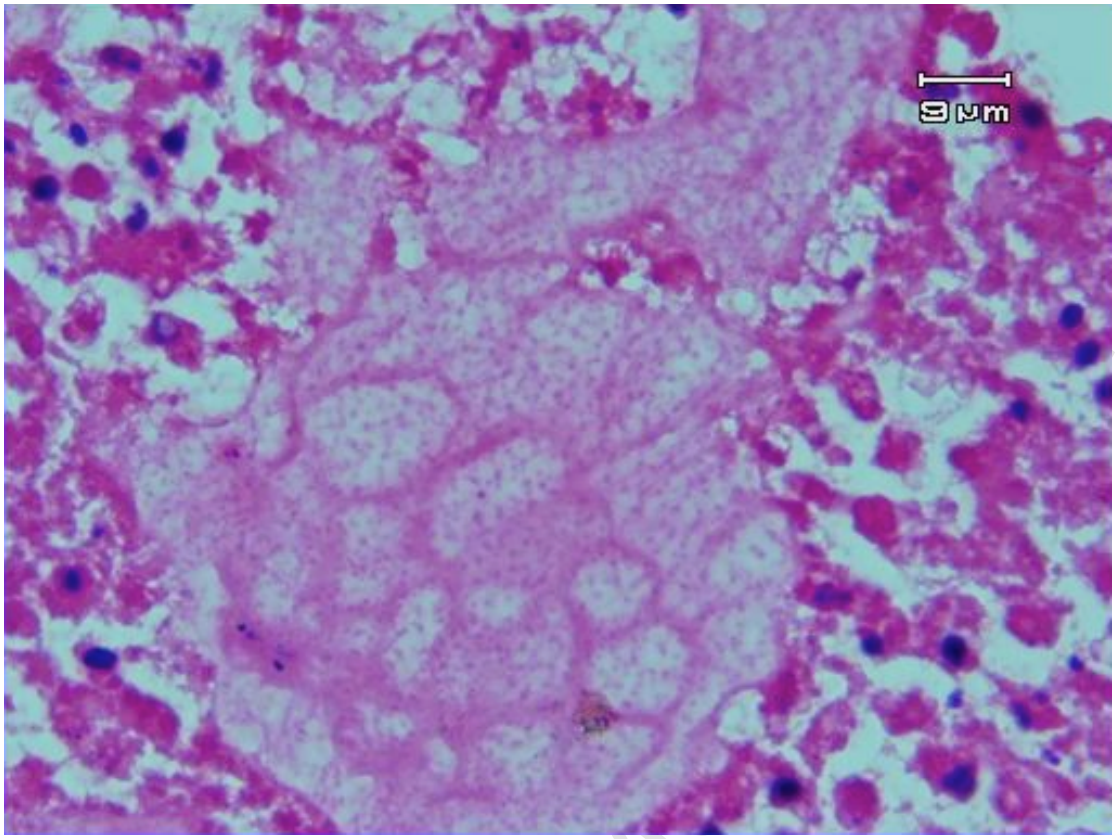

Fig. 8. Gram stain (x 1000) of an explanted lung with a biofilm of *P. aeruginosa* surrounded by numerous polymorphonuclear leukocytes. The patient is a 41 year old cystic fibrosis male who has suffered from chronic mucoid *P. aeruginosa* lung infection for 28 years and has been treated with 114 2-weeks anti-pseudomonas antibiotic treatment courses (chronic suppressive maintenance therapy, total: one kg tobramycin, 10 kg betalactam antibiotics and 1 kg colistin). He had developed 46 precipitating antibodies against *P. aeruginosa* (normal: 0-1).