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1 **Antibiotic resistance of bacterial biofilms**

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21

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- 31 • Foreign body infections

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38 ABSTRACT

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

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

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

58

59 **1. Introduction**

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

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

66

67 **2. The occurrence and architecture of bacterial biofilms**

68 Foreign body infections are characterized by biofilm growth of the bacteria on the
69 outer and/or inner surface of the foreign body (Table 2). Biofilm growth also occurs
70 on natural surfaces such as teeth (3), heart valves (endocarditis), (4) in the lungs of
71 cystic fibrosis (CF) patients causing chronic bronchopneumonia (2), in the middle ear
72 in patients with persistent otitis media (5), in chronic rhinosinusitis (6), in chronic
73 osteomyelitis and prosthetic joint infections (7) (8) (9), in intravenous catheters and
74 stents (10) and in chronic wounds (11) (12)(Fig. 1 A & B). The microbes in biofilms
75 are kept together by a self-produced biopolymeric matrix. The matrix contains
76 polysaccharides, proteins and DNA originating from the microbes and the bacterial
77 consortium can consist of one or more species living in a sociomicrobiological way
78 (1, 13) (2, 14). The matrix is important since it provides structural stability and
79 protection to the biofilm. The development of bacterial biofilms over time has been
80 intensively studied *in vitro* by confocal scanning laser microscopy employing *green*
81 *fluorescent protein (gfp)*-tagged bacteria. This technique has been combined with
82 advanced *in silico* image-analysing to produce 3-D images of biofilm (15, 16) (17).
83 As an example, *P. aeruginosa* produce a mature *in vitro* biofilm in 5-7 days (Fig. 2).

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

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

112 testing therefore gives misleading results which do not reflect the increased resistance
113 of the bacteria when living in biofilms. The minimal inhibitory concentration (MIC)
114 and minimal bactericidal concentration (MBC) of antibiotics to biofilm growing
115 bacteria may be up to 100 – 1000 fold higher compared to planktonic bacteria (21)
116 (22, 23). Methods to test biofilm growing bacteria have therefore been developed but
117 their clinical relevance as regards prediction of clinical successful therapy awaits
118 confirmation (22, 24, 25).

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

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

131 **4. Mutators**

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

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

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

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

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

169 The hypermutability of bacteria in biofilms promotes the emergence of mutations
170 conferring antibiotic resistance which will be selected for by the repeated antibiotic
171 courses administered in order to maintain the lung function of CF patients.

172 Development of resistance to all classes of antibiotics during the chronic lung
173 infection in CF has been documented (43). Resistance to beta-lactam antibiotics occur
174 due to mutations in regulatory genes of the beta-lactamase production leading to the
175 occurrence of isolates with stable or partially-stable derepressed production of AmpC
176 beta-lactamase(44). Resistance to ciprofloxacin of CF *P. aeruginosa* isolates was
177 shown to be mediated by mutations in *gyrA* and alterations in the two efflux systems
178 MexCD-OprJ and MexEF-OprN and resistance to tobramycin was overexpression of
179 the MexXY-OprM multi-drug efflux pump (45) (46). Resistance to colistin was
180 shown to occur due to mutations in the *pmr* system involved in the LPS structure
181 (47).

182

183

184 5. Chromosomal beta-lactamase and biofilm matrix components

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

201

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

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

214 The source of β -lactamase in biofilms has been considered to be from a layer of lysed
215 bacteria due to exposure to an antibiotic, with release of defensive enzymes into the
216 extracellular space. We have shown that the source of β -lactamase in biofilm may
217 also be the membrane vesicles (MVs) containing beta-lactamase liberated by resistant
218 *P. aeruginosa* bacteria (57)(58)(54) and we have shown, that high level of free
219 chromosomal beta-lactamase is present in CF sputum (58). We have also shown that
220 strong inducers like imipenem will induce the beta-lactamase through all the bacterial
221 layers while poorer inducers like ceftazidime will influence just the superficial layers
222 of the biofilm, probably due to the inactivation of the antibiotic by beta-lactamase (59,
223 60)(61)(58) (Figure 3).

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

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

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

252

253 **6. Tolerance, adaptive resistance and efflux pumps**

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

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

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

273

274 **7. High cell density and quorum sensing**

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

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

290

291 **8. Quorum sensing inhibitors (QSI)**

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

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

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

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

335

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

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

353 The recommended method for treatment of chronic *P. aeruginosa* biofilm

354 infection is chronic suppressive antibiotic therapy (2)(110) (Fig. 8) which is started
355 when the chronic infection is diagnosed (continuous colonization at the monthly
356 bacteriological examination for 6 months and/or increased level of antibodies against
357 *P. aeruginosa* (111)(112) . The chronic suppressive therapy consists of daily
358 nebulized colistin or tobramycin for the rest of the patient's life combined with either
359 regular 2-week courses every 3 months of intravenous anti-pseudomonas antibiotics
360 (combination therapy of 2 antibiotics: tobramycin or colistin + ceftazidime, or
361 piperacillin/tazobactam, or carbapenem, or aztreonam, or ciprofloxacin) or ad hoc
362 intravenous therapy when clinical deteriorations occur (110). Additionally, DNase is
363 inhaled every day to reduce the viscosity of the DNA-containing sputum (105), and
364 daily oral azithromycin is given to the patients as mentioned above (95). The chronic
365 suppressive therapy (maintenance therapy) has successfully been able to maintain the
366 pulmonary function or slow the decline of the pulmonary function and prolong the life
367 of the patients for many years (113). The side-effects of the maintenance therapy is
368 high level of conventional resistance mechanisms in the persisting strains and high
369 level of allergy to the beta-lactam antibiotics (114) (43). Similar principles, a)
370 systemic or local prophylactic use of antibiotics to prevent biofilm formation, b) early
371 aggressive eradication therapy to eradicate planktonic growth or early biofilm
372 formation and c) chronic suppressive antibiotic therapy to maintain the function of a
373 inserted foreign body are gradually being introduced in other area of biofilm infection
374 (100) (8) (9). Possibly other approaches taken advantage of the use of QSI may
375 further improve the management of biofilm infections.

376

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378 None.

379

380 Conflict of interest statement

381 None to declare.

382

383

384

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Table 1. Some general features of biofilm infections in humans compared to acute planktonic infections and superficial colonization/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 infections	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 response	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 infection	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 $\times 100$ and $\times 1000$. 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 (*PampC-gfp*): 6-day-

old biofilm exposed to 100 $\mu\text{g/mL}$ ceftazidime for 4 h. Detection level of the monitor, 10 $\mu\text{g/mL}$ 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 \times 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 \times 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 \times 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 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ ciprofloxacin and 25 $\mu\text{g}/\text{mL}$ colistin. Reproduced from [71] with permission.

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

tobramycin 10 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ tobramycin-treated wild-type; (c) 20 $\mu\text{g}/\text{mL}$ tobramycin-treated wild-type; (d) untreated $\Delta\text{lasRrhIR}$ mutant; (e) 10 $\mu\text{g}/\text{mL}$ tobramycin-treated $\Delta\text{lasRrhIR}$ mutant; and (f) 20 $\mu\text{g}/\text{mL}$ tobramycin-treated $\Delta\text{lasRrhIR}$ 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 $\mu\text{g}/\text{mL}$ 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 ($\times 1000$) 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 (slgA)
Inflammation	Yes	No	Yes	No
Biofilm-specific antigens	No and yes—seldom, e.g. <i>Pseudomonas aeruginosa</i> alginate	No and yes—seldom, e.g. <i>P. aeruginosa</i> 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; slgA, serum immunoglobulin A.

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.

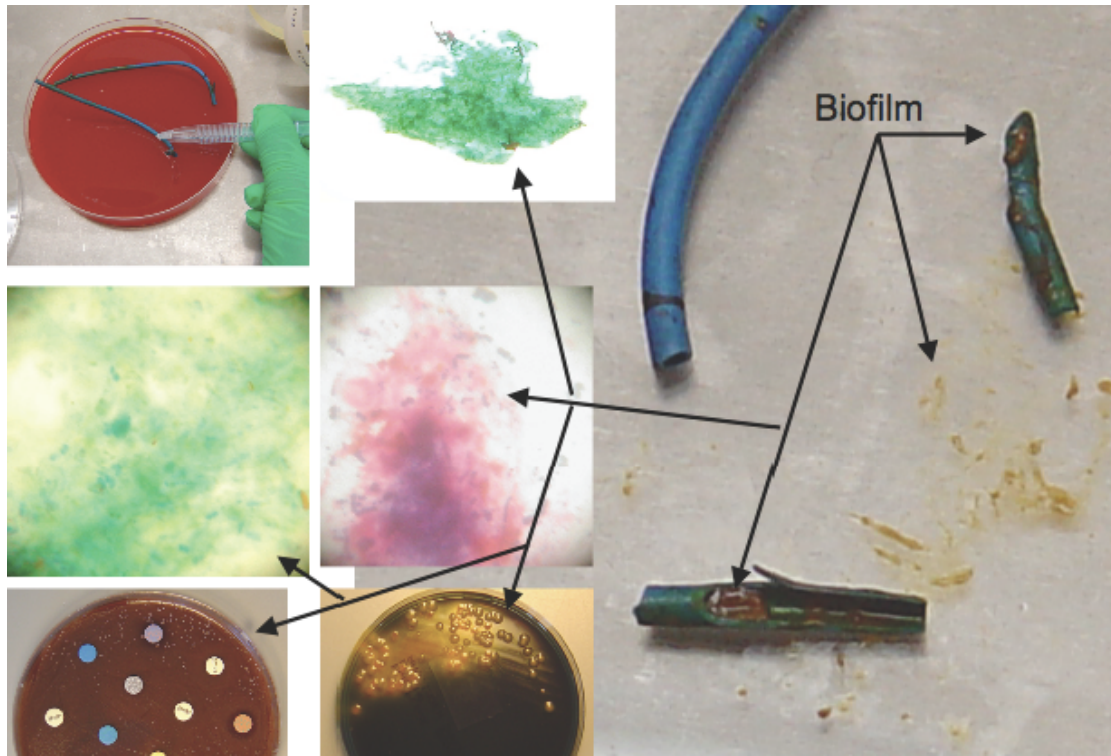


Fig. 1B.

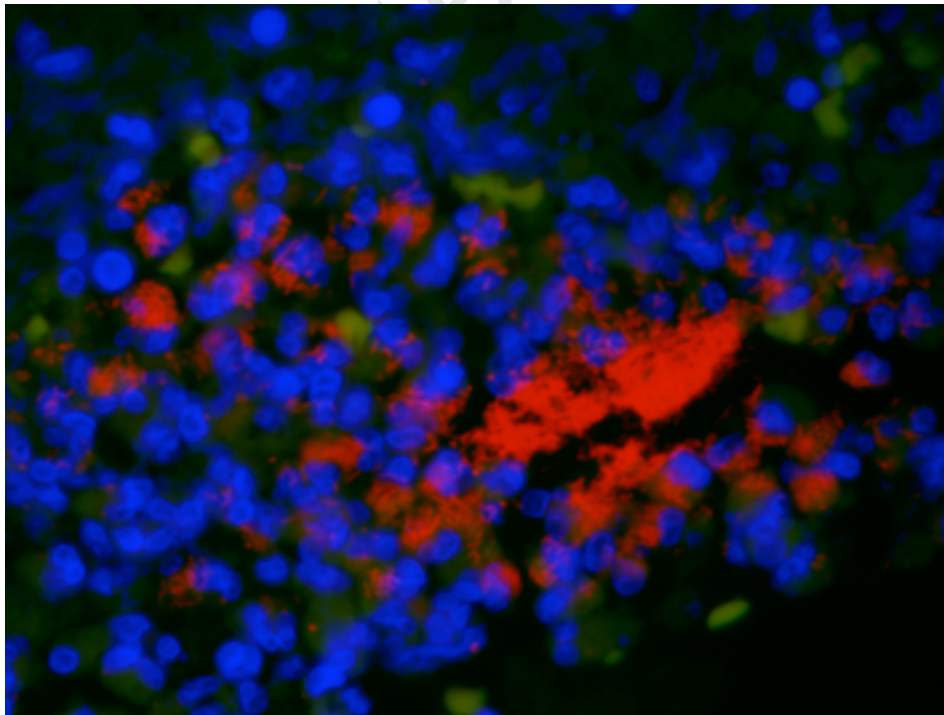


Figure 1. *A.* Example of a biofilm on a biliary stent which became focus of repeated incidents of sepsis which ultimately was lethal. DNA typing (pulsed field gel electrophoresis) showed that it was the same clonal type of *E. coli* in the biofilm and in the blood. In spite of relevant antibiotic therapy 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.

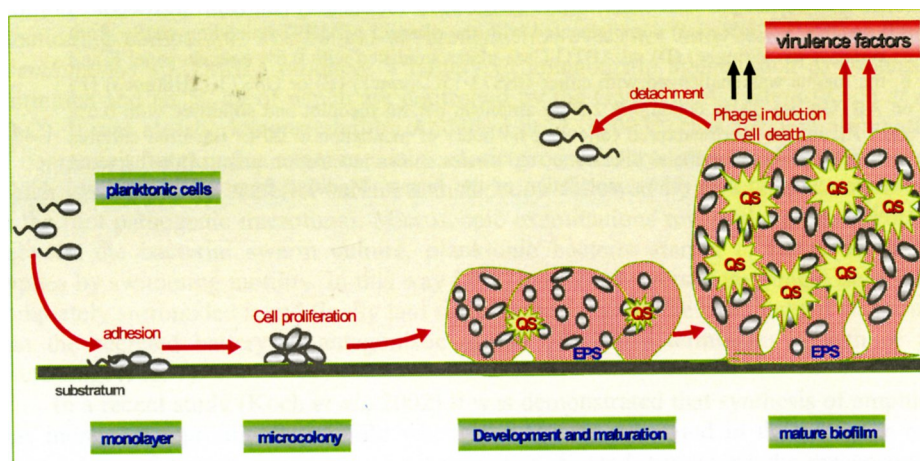


Figure 2. Time-course of formation of a *P. aeruginosa* biofilm based on *in vitro* experiments with *green fluorescent protein*-tagged *P. aeruginosa*, which grow in a *flow-cell* and is examined with a confocal scanning laser microscope. QS indicates cell-to-cell communication and EPS is hydrated extracellular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

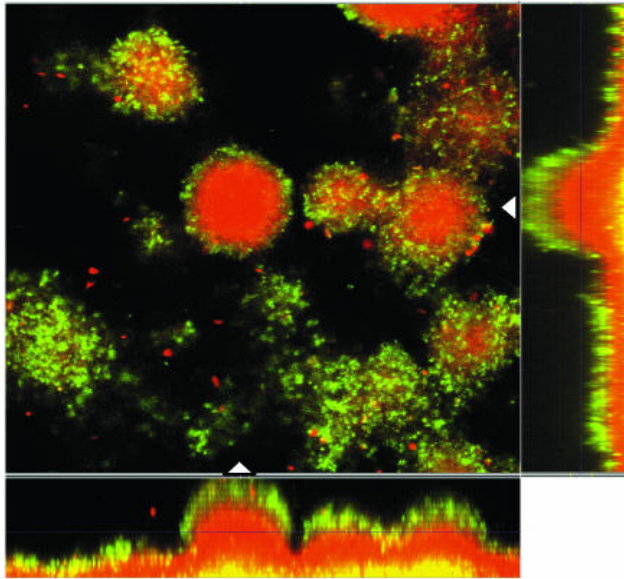


Figure 3A. Induction of beta-lactamase in *P. aeruginosa* biofilm. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) when the promoter of the AmpC beta-lactamase is induced (*PampC-gfp*): 6 days old biofilm exposed to 100 $\mu\text{g/ml}$ ceftazidime for 4 h. Detection level of the monitor: 10 $\mu\text{g/ml}$ ceftazidime.

Reproduced from (116) with permission.

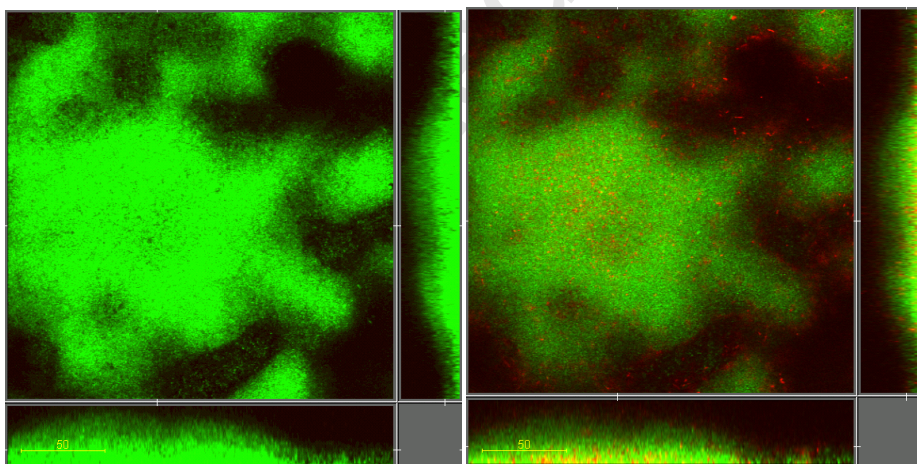


Figure 3B. Treatment of *P. aeruginosa* biofilm with beta-lactams. *P. aeruginosa ampD⁻* (levels AmpC beta-lactamase (mU) :1050 basal, 4255 induced) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with 10 times MIC of ceftazidime. Propidium iodide was added after day 6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

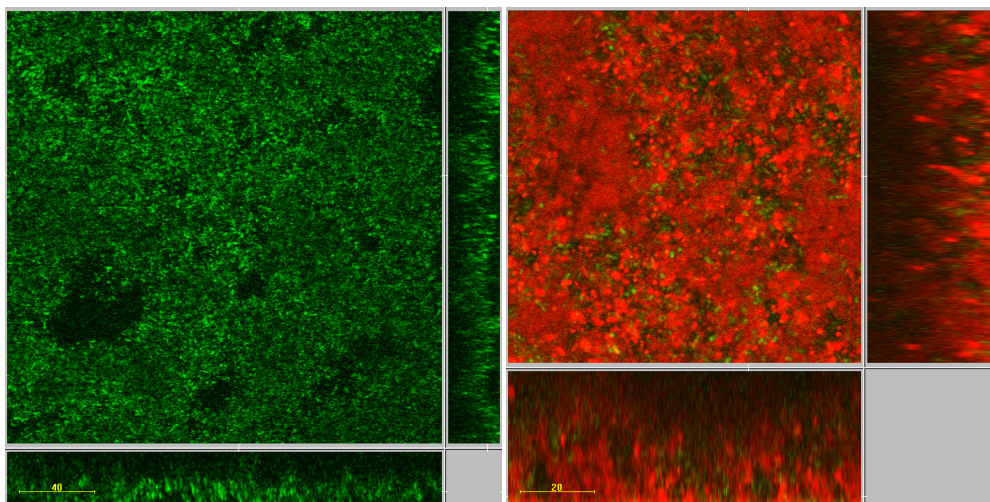


Figure 3C. Treatment of *P. aeruginosa* biofilm with beta-lactams. *P. aeruginosa ampD+* (levels AmpC beta-lactamase (mU): basal 3, induced 175) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with 10 times MIC of ceftazidime. Propidium iodide was added after day 6 to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

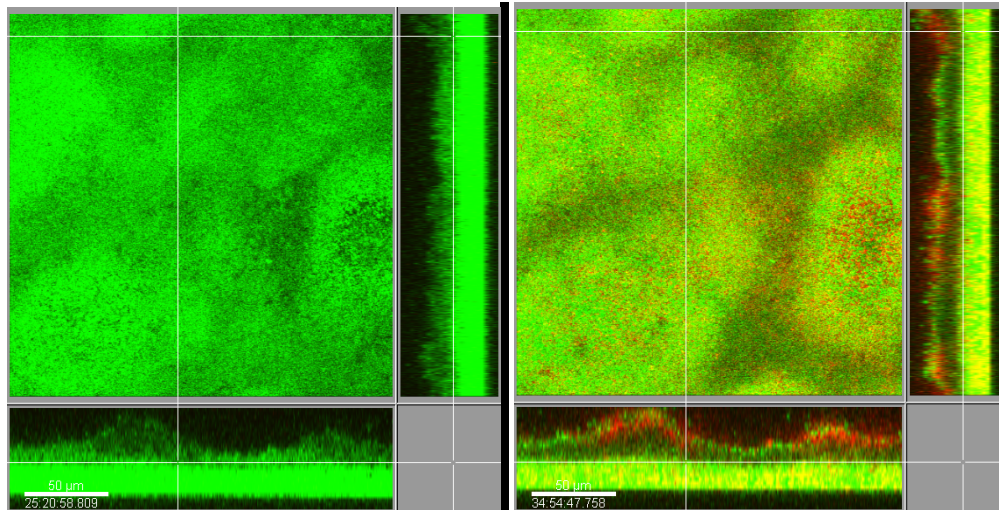


Figure 3D. Treatment of *P. aeruginosa* biofilms with a combination of ceftazidime and aztreonam. *P. aeruginosa ampD⁻* (levels AmpC beta-lactamase (mU) :1050 basal, 4255 induced) expressing green fluorescent protein (gfp) as a tag. 7 days old biofilm treated with a combination of ceftazidime and aztreonam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

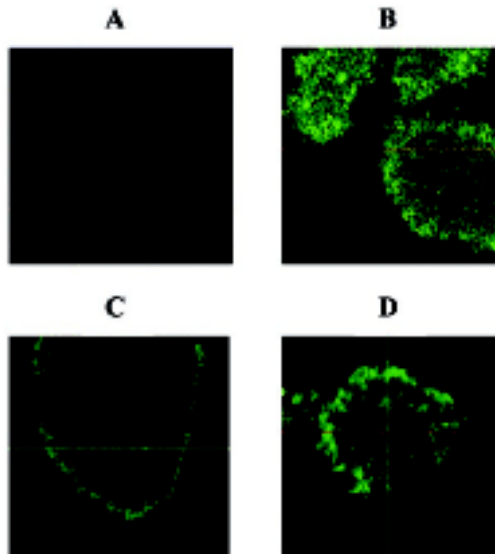


Figure 4 Induction of alginate in *P. aeruginosa* biofilms treated with sub-MIC concentrations of imipenem (62) A. *P. aeruginosa* PAO1 not exposed to antibiotics; B. PDO300 (a PAO1 derivative constitutively expressing alginate) not exposed to antibiotics; C. PAO1 exposed to imipenem for 18 hours; D.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

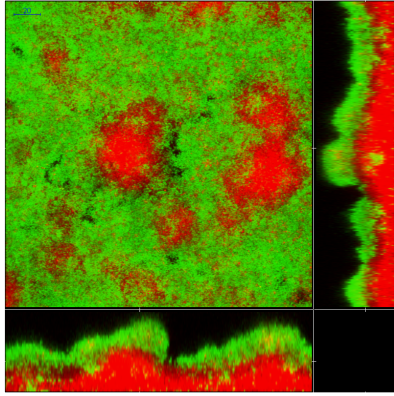


Figure 5A. Treatment of *P. aeruginosa* biofilms with colistin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag were grown as biofilms in flow-chambers for 4 days. Propidium iodide was added after day 4 to continuously monitor the killing of the biofilm by colistin. The picture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

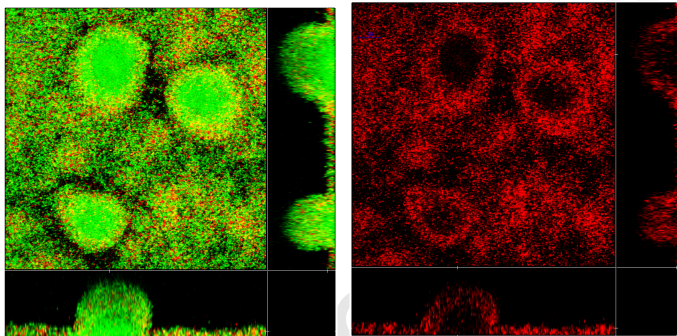


Figure 5 B. Treatment of *P. aeruginosa* biofilms with ciprofloxacin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag was grown as biofilm in a flow-chamber for 4 days and was treated for 2 days with ciprofloxacin 10 $\mu\text{g}/\text{ml}$ (A). Propidium iodide was added after day 4 to continuously 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.

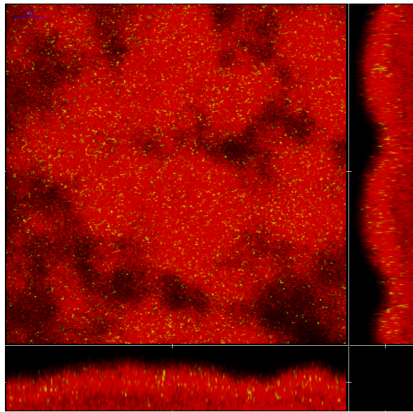


Figure 5C Treatment of *P. aeruginosa* biofilm with a combination of ciprofloxacin and colistin. *P. aeruginosa* PAO1 expressing green fluorescent protein (gfp) as a tag was grown as biofilm in flow-chambers for 4 days. Propidium iodide was added after day 4 to continuously monitor the killing of the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

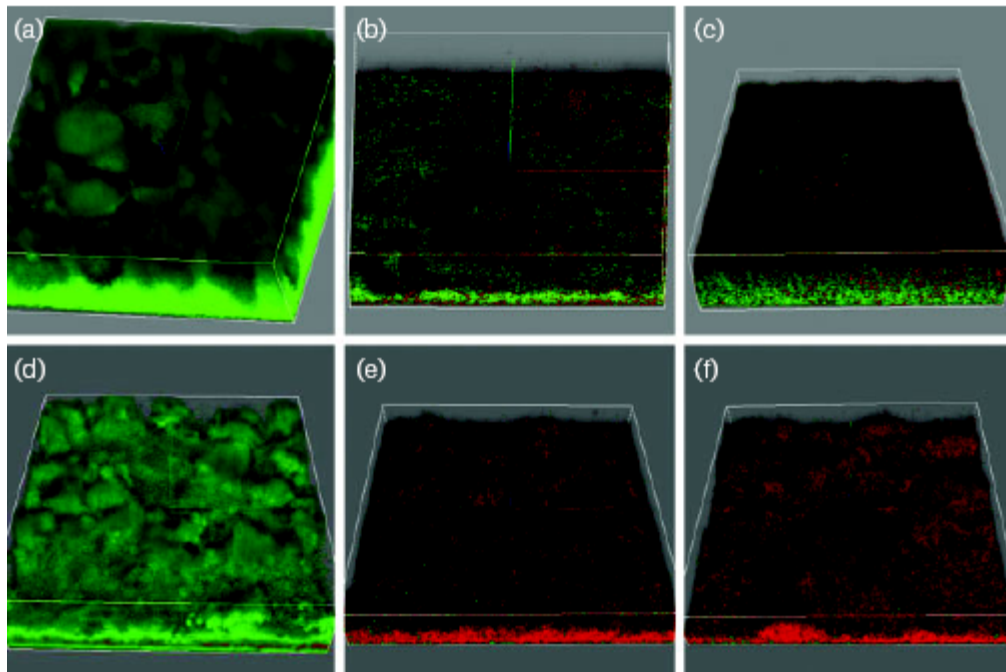


Figure 6. Treatment of *P. aeruginosa* biofilm with tobramycin. Wild-type PAO1 and Δ lasRrhIR mutant, both expressing green-fluorescent protein (gfp) as a tag were grown as biofilms in flow-chambers for 3 days. On day 3 tobramycin 10 μ g/ml and 20 μ g/ml were added. Propidium iodide was added after day 3 to continuously 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 μ g/ml wild-type, (c) 20 μ g/ml wild-type, (d) untreated Δ lasRrhIR mutant, (e) 10 μ g/ml Δ lasRrhIR mutant, (f) 20 μ g/ml Δ lasRrhIR mutant. Reproduced with permission from (74) .

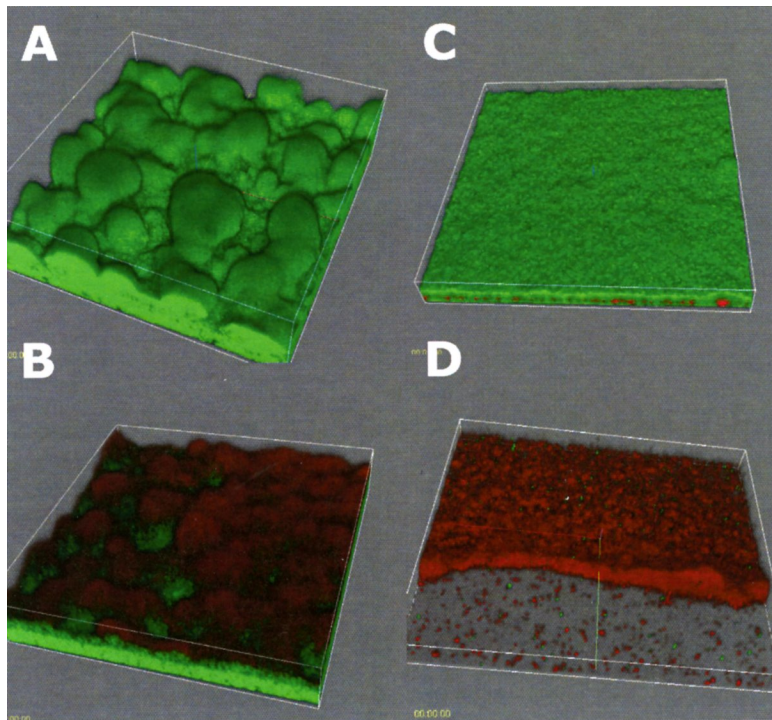


Fig. 7. A 4 day old biofilm formed by *green fluorescent protein*-tagged *P. aeruginosa* cultured in absence (A and B) or presence (C and D) of 2% garlic extract. The biofilms B and D were treated on day 3 with 340 $\mu\text{g/ml}$ tobramycin for 24 h. The biofilms were then stained with LIVE/DEAD *BacLight* 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.

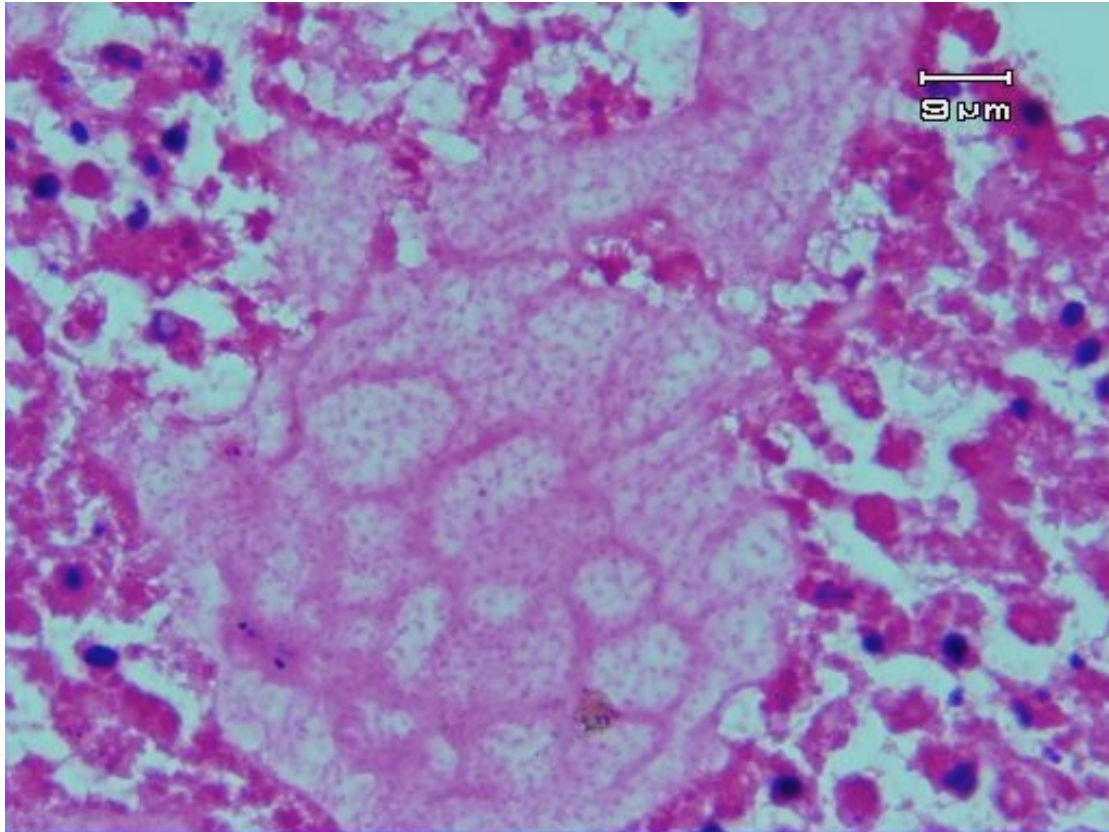


Fig. 8. Gram stain (x 1000) of an explanted lung with a biofilm of *P. aeruginosa* surrounded by numerous polymorphonuclear leukocytes. The patient is a 41 year old cystic fibrosis male who has suffered from chronic mucoid *P. aeruginosa* lung infection for 28 years and has been treated with 114 2-weeks 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).