Corso Precongressuale A
«Microbiologia della Fibrosi Cistica e altre patologie croniche, tra fenotipi, antibiotipi e biofilm"

Biofilm ed antimicrobici: una fortezza inespugnabile ?

(Biofilm and antimicrobials: an impregnable fortress?)

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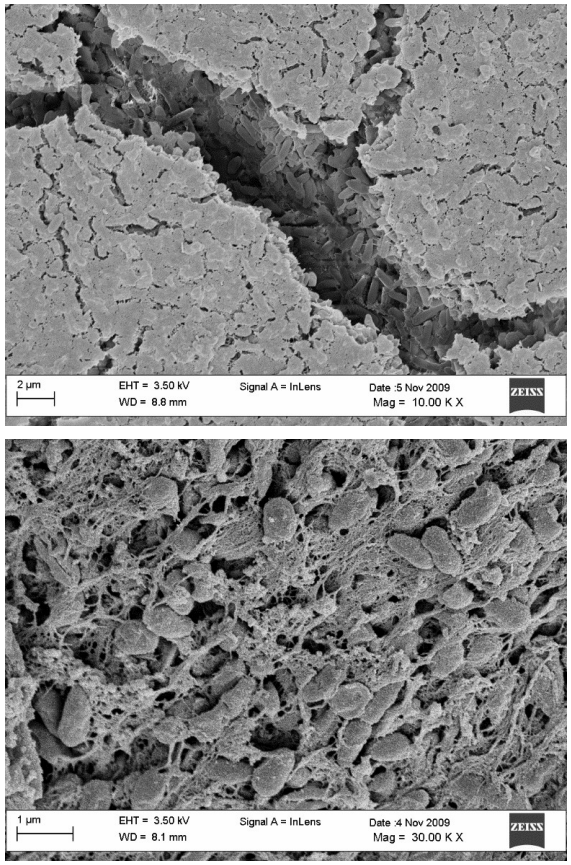
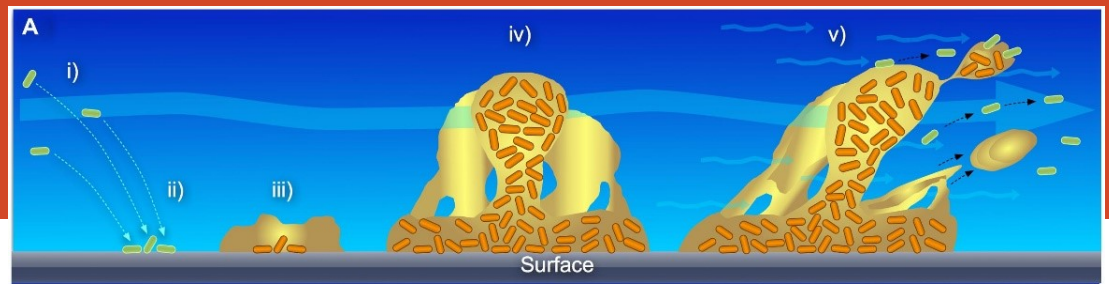
Biofilm: a microbial «*consortium*»

- Although the term **biofilm** was introduced in 1981, bacterial aggregation has been observed in the 'scurf of the teeth' by Anthony van Leeuwenhoek (published in 1684).
- Biofilm is generally known as community (*consortium*) of microbes, established in a **three-dimensional structure**, that can be attached - to abiotic (prosthetic devices) or biotic (epithelia) surfaces – or floating.
- In these aggregates, bacteria are physically joined together and they produce an **extracellular matrix** that contains many different types of extracellular polymeric substances (EPS) including exopolysaccharides, extracellular DNA (eDNA), RNA, proteins, and lipids.[1-3]
- Microbes in a biofilm can communicate with each other by chemical signals, produced by cells and passed through their outer membranes (**QS, Quorum-Sensing**); communication can be intra- and inter-species and modulates virulence traits, such as biofilm formation.



P. aeruginosa biofilm (Pompilio et al, DMID 2016)

Biofilm formation

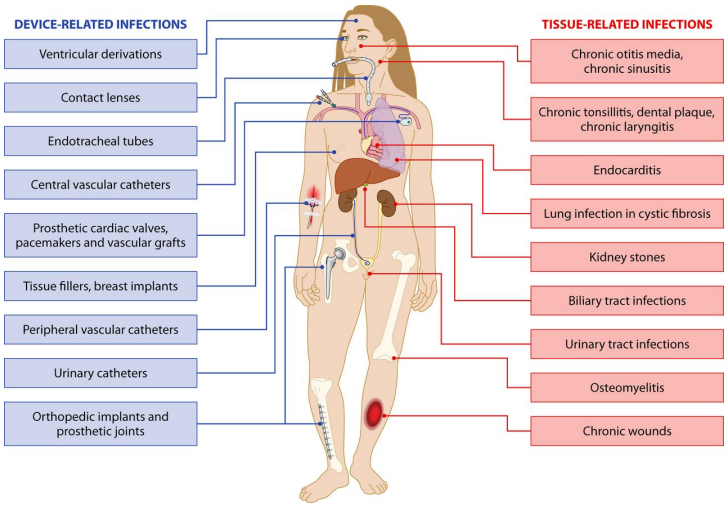


The planktonic-to-biofilm cells transition is a complex and highly regulated process, dependent on the expression of a specific genetically encoded program.[2,4] It can be divided into three different stages:[5-7]

1. **attachment**: initial reversible binding of bacteria, then irreversible attachment; bacteria move over the moist surface by twitching,[8] forming bacterial microcolonies by clonal growth.
2. **maturation:** mature biofilms show a characteristic network of mushroom-like structures and open voids that develop over time. A mushroom is composed of a stalk (formed by clonal growth) and a cap (formed by motile bacteria climbing the stalks), whose formation is influenced by nutrient availability and other environmental conditions.[9] Subsequent adaptation to a microenvironment characterized by steep chemical gradients and mass transfer limitations for O_2, nutrients, and substrates.
3. **dispersion**: single cells detach on a genetically programmed process or on enzymes such as dispersin B,[10] whereas cell clusters can also be removed by hydrodynamic shear forces,[7] and/or by prophage-mediated cell death.[11,12] Dispersal of biofilms during chronic infection can cause an acute bloodstream infection, as in the case of *Burkholderia* species in CF paients.[13]

Clinical relevance of biofilm

- Bacteria in biofilms are **inherently more resistant** - up to 1.000 times - to various **antimicrobials** (antibiotics and disinfectants) and to the **host immune response** than their planktonic counterparts.[7,14]
- This leads to **chronic infection** which threatens many lives worldwide.[7] Microscopic investigations of numerous chronic infections have in fact revealed that infecting bacteria are physically aggregated in biofilm.
- However, biofilms are not always bad, and are positively used in many applications. In the bioremediation process, they degrade many toxic contaminants and hazardous materials that are generated from various industrial processes.[15]

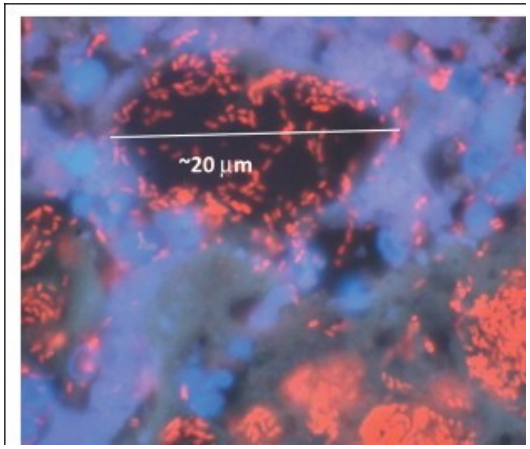


Lebeaux et al, Microbiol Mol Biol Rev. 2014:510

The major hallmarks of *in vivo* biofilms are thus a coherent cluster of aggregated bacteria embedded in a matrix, which tolerate the host defense and high concentrations of antimicrobial agents even over longer times.

Biofilm in CF patients

- The first observation linking the etiology of a persistent (chronic) infection to the aggregation of bacteria was reported in the 1970s in the lungs of patients suffering from cystic fibrosis (CF).[16]
- *P. aeruginosa* is notorious for causing pneumonia in the CF patients, where it is the primary cause of death.[17]
- *P. aeruginosa* is able to **persist in CF lung by switching to the biofilm mode** of growth consisting of small cellular aggregates encased in EPS that induce an ongoing and self-reinforcing co-activation of the innate and adaptive immune response leading to persistent inflammation during chronic lung infection.[18]
- Biofilm cells are tolerant to the inflammatory defense mechanism, to the aerobic respiratory zone and to the conductive zone of the lungs which contain anaerobic sputum, and to antibiotic therapy.

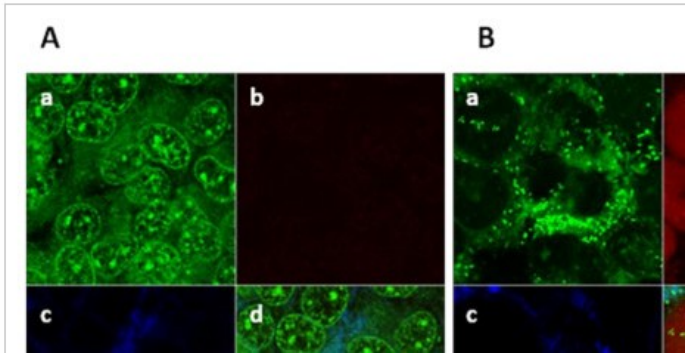


Biofilm aggregates of *P. aeruginosa* in a chronic infected cystic fibrosis (CF) lung. Using a specific *P. aeruginosa* PNA fluorescence *in situ* hybridization (FISH) probe, the bacteria are visualized in red, whereas the inflammatory cells surrounding the biofilm patches are counterstained with DAPI (blue). *Bjarnsholt, Trends in Microbiology 2013;21:466*

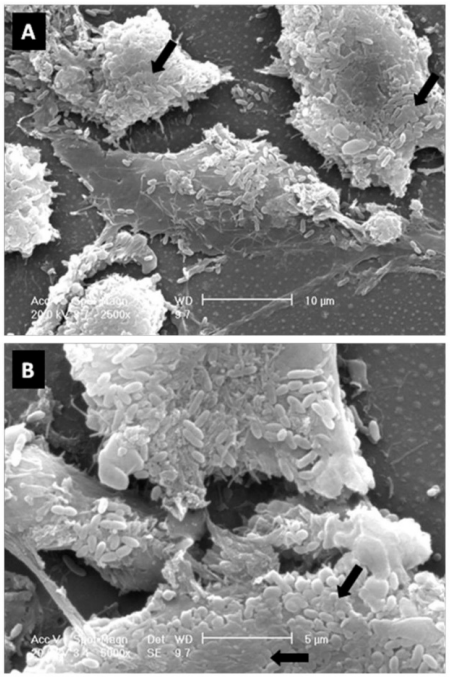
- This **prolonged inflammatory response**, dominated by recruited polymorphonuclear neutrophils and not the bacteria *per se*, causes tissue damage, necrosis of the lung tissue, and eventual death of the patient.[16,19,20]
- The biofilm strategy is also used by other CF pathogens (*Burkholderia, Staphylococcus, Stenotrophomonas* spp)

RESEARCH ARTICLE

Adhesion to and biofilm formation o bronchial cells by *Stenotrophomona* isolates from cystic fibrosis patients



CLSM observation of 24 h-biofilm by *S. maltophilia* OBGTC9 CF strain on IB3-1 cell monolayer. A) uninfected (control), and B) OBGTC9-exposed IB3-1 cell monolayer. Image capture was set for visualization of: (a) green fluorescence (Syto-9, live cells); (b) red fluorescence (propidium iodide (dead cells); (c) blue fluorescence (Con-A, extracellular matrix); and (d) co-localization.



SEM observation of 24 h-biofilm formed by CF *S. maltophilia* OBGTC9 CF strain on IB3-1 cell monolayer. Microcolony formation indicates biofilm formation. Arrows show the presence of extracellular matrix.

RESEARCH ARTICLE

Phenotypic and genotypic characteriza *Stenotrophomonas maltophilia* isolates f patients with cystic fibrosis: Genome d biofilm formation, and virulence

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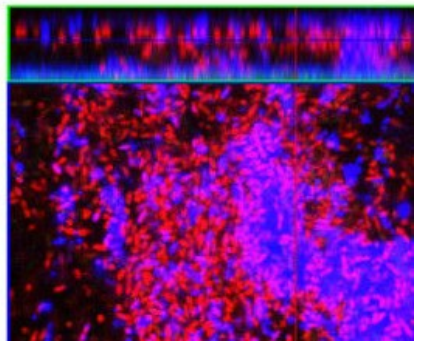


CLSM examination of *S. maltophilia* Sm192 24h-biofilm. Orthogonal images showed a biofilm with a multilayered structure (red, propidium iodide-stained) embedded in an abundant extracellular polymeric substance (blue, concanavalin A-stained). Magnification, ×100.

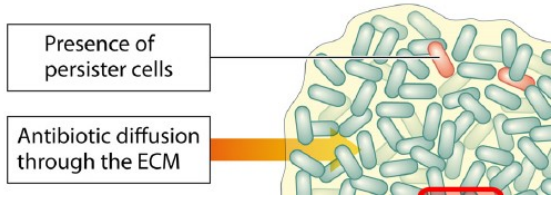
Reduced susceptibility of biofilm to antibiotics:
a problematic mixture of tolerance and resistance

Challenges in antimicrobial treatment of biofilms
Antimicrobial tolerance of biofilms

Related to biofilm mode of growth, tolerance reverts after biofilm-to-planktonic transition.

Multifactorial, since it is attributed to:

- **limited penetration of the antibiotics in the matrix**
  - electric interaction with EPS/e-DNA (i.e. aminoglycosides) or enzymatic inactivation (i.e. β-lactamases)
- **differential physiological activity**
  - low metabolic activity (especially aminoglycosides, fluoroquinolones) as a consequence of restricted bacterial growth due to limited O_2 and nutrient penetration (high-to-low gradient)
- expression of **biofilm-specific genes**
  - *ndvB* in *P. aeruginosa* encodes periplasmic glucans sequestring tobramycin
- presence of «**persisters**»
  - very low fraction (<0.1%) of cells differentiated into a dormant state; also resistant to antibiotics that kill non-growing cells



Challenges in antimicrobial treatment of biofilms
In vivo «adaptative» tolerance

***In vitro* tolerance is supplemented *in vivo* by a complex of conditions:**

- presence of the **immune system**
- biofilm formation in areas with **low O_2 tension**
  - in sputum and sinus secretion of chronically infected CF patients, neutrophils consume O_2 creating anaerobic conditions and affecting both ROS-dependent effect of bactericidal antibiotics (fluoroquinolones, aminoglycosides, β-lactams) and O_2-mediated transport across the membrane (aminoglycosides)
- **reduced antibiotic concentration** at the infection site
  - different compartments between antibiotic and biofilm: 1st (blood) – 2nd (tissue) – 3rd (biofilm)
  - concentration dependent on biofilm (size and location), as well as individual drug metabolization (large variation in the pharmacokinetics of clarithromycin among CF patients)
  - antibiotics at sub-MICs select resistant populations (due to increased mutagenesis)
- **gene expression modulation**
  - upregulation (efflux pumps, alginate production, β-lactamases) in the presence of antibiotics, downregulation when antibiotics are metabolized

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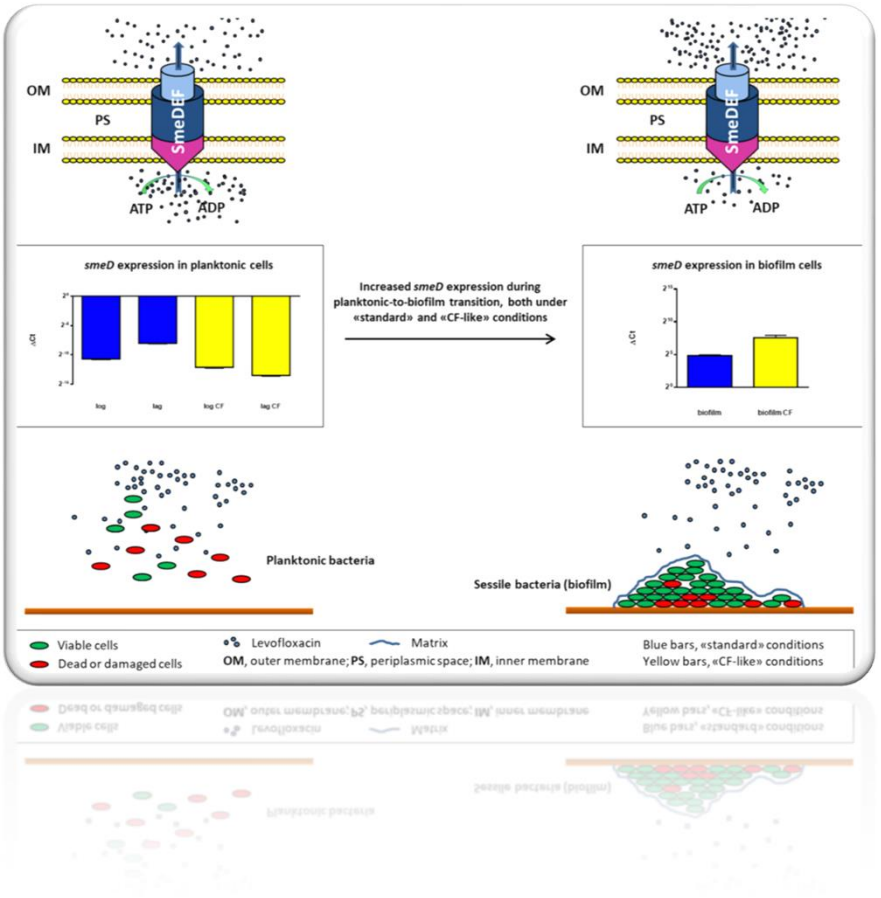
doi: 10.1093/femsle/fnw145
Advance Access Publication Date: 29 May 2016
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

In vitro activity of levofloxacin against planktonic and biofilm *Stenotrophomonas maltophilia* lifestyles under conditions relevant to pulmonary infection in cystic fibrosis, and relationship with SmeDEF multidrug efflux pump expression

Arianna Pompilio[1,2], Valentina Crocetta[1,2], Fabio Verginelli[2,3] and Giovanni Di Bonaventura[1,2,*]



- **multidrug efflux pump SmeDEF overexpression during planktonic-to-biofilm transition causes increased resistance of *S. maltophilia* biofilm to levofloxacin**
- **it occurs under 'CF-like' conditions only, suggesting that one or more components of CF sputum improve *smeD* expression**

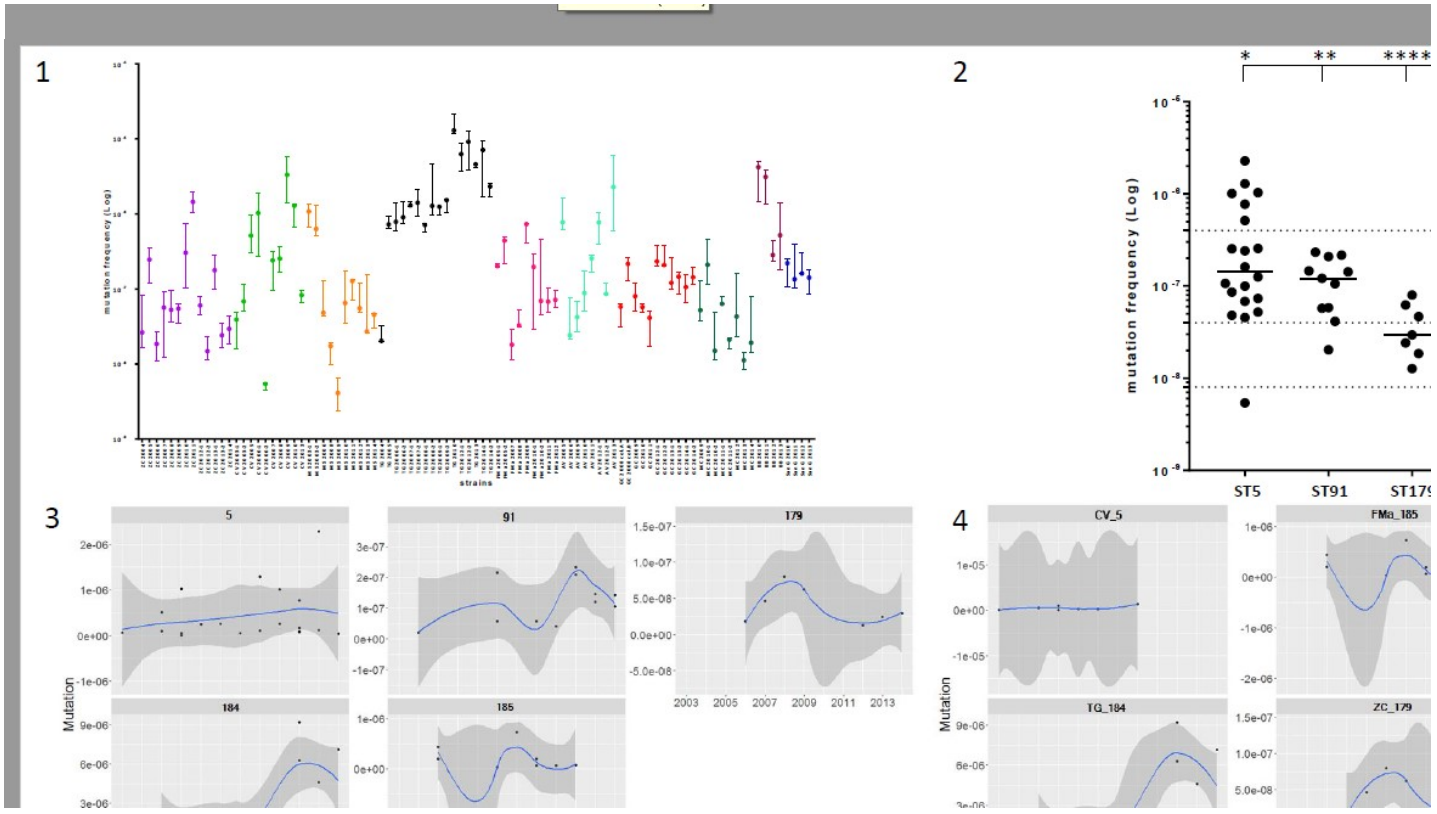
Challenges in antimicrobial treatment of biofilms
Antimicrobial resistance of biofilms

Not related to the biofilm mode of growth (also dysplayed by planktonic cells), **resistance is due to mutations**

- acquisition of **chromosomal mutations**, causing:
  - upregulation of efflux pump system
  - downregulation of enzymes (i.e. AmpC β-lactamase)
  - permeability changes
  - altered antibiotic targets (PBPs)
- accumulation of mutations can be facilitated by **hypermutator microorganims**:
  - 100- to 1000-fold increased mutation rate, due to defects in DNA repair or error avoidance systems (MMRS, GO system, prevention of oxidative damage produced by ROS)
  - isolated in 30-60% of CF patients, acute-to-chronic transition leads to increased prevalence (0 to 65%) of mutator strains (*P. aeruginosa*, *S. maltophilia*)
  - antibiotic therapy also selects for hypermutators

frontiers in Microbiology

Evolution of *Stenotrophom* *maltophilia* in Cystic Fibros over Chronic Infection: A G and Phenotypic Population

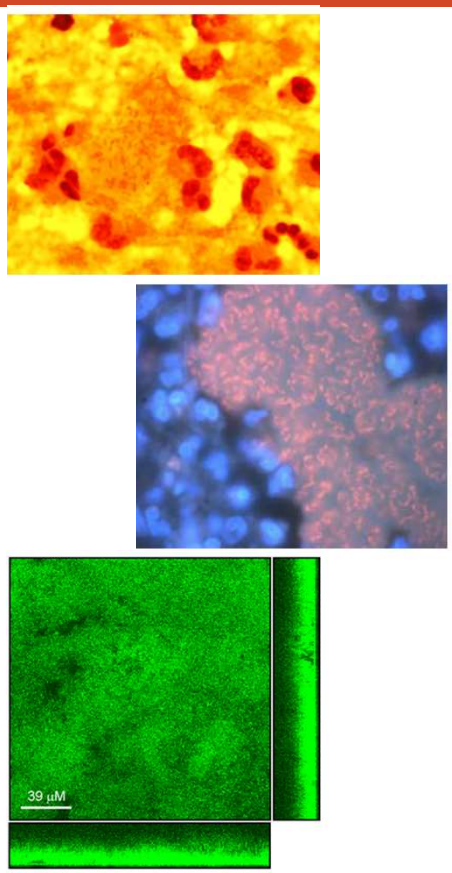


Diagnosis of biofilm-related infections

Diagnosis of biofilm-based infection
Microscopy

- samples: sputum, mucus from paranasal sinuses, lung tissue
- **brightfield microscopy**: Gram (Hematoxylin-Eosin, Ziehl-Neelsen)-stained smears
- **fluorescent microscopy**:
  - FISH: probe signal dependent on the number of ribosomes in each bacterial cell; dormant/slow growing bacteria may therefore show weak fluorescence
  - PNA-FISH: more susceptible and specific
  - CLSM: the most direct way of demonstrating biofilms in clinical specimens, although it is time-consuming and requires highly specialized training

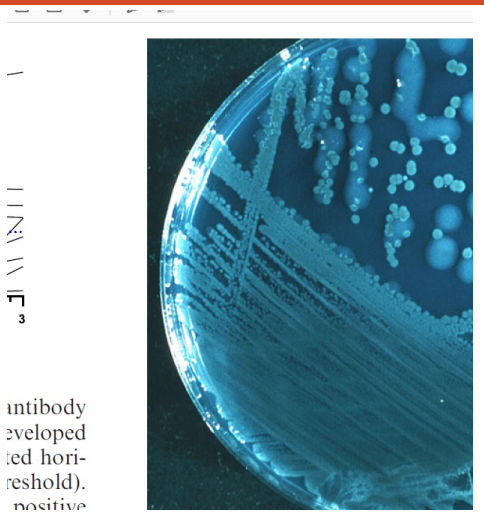
At microscopic observation, biofilms are small aggregates of bacteria (4–100 µm) embedded in a polysaccharidic matrix dominated by alginate (stained by Alcian blue or Calcofluor), and surrounded by numerous polymorphonuclear leukocytes



Høiby N, et al. APMIS 2017;125:339
Bjarnsholt et al, Trends Microbiol 2013

Diagnosis of biofilm-based infection
Mucoid phenotype

- at **culture analysis**, *P. aeruginosa* colonies can appear as mucoid or rough
- mucoid phenotype is due to hyperproduction of alginate, secondary to mutations occurred in *mucA*
- (frequent) simultaneous presence of non-mucoid colonies of the same genotype due to additional mutations in *algT* (*algU*)



Høiby N, et al. APMIS 2017;125:339

little actually reaches the respiratory zone, since the 3 minutes one to thre

Table 1 Important properties of mucoid and nonmucoid phenotypes of *Pseudomonas* tract of cystic fibrosis patients[a]

Property	Mucoid phenotype
Location in the lungs	Respiratory zone and conductive zone i
Biofilm formation *in vitro*	Yes

Høiby N. BMC Med 2011

Diagnosis of biofilm-based infection
Antibody response

- **crossed immunoelectrophoresis, ELISA**
- *P. aeruginosa* biofilms: detection of **IgG** (serum) and/or **sIgA** (saliva and secretions from the paranasal mucosal) to antigens (proteins, LPS, alginate)
- in case of other biofilms than *P. aeruginosa* (*S. maltophilia, B. multivorans, A. xylosoxidans*), there is no alginate present and only serum IgG antibodies have been used

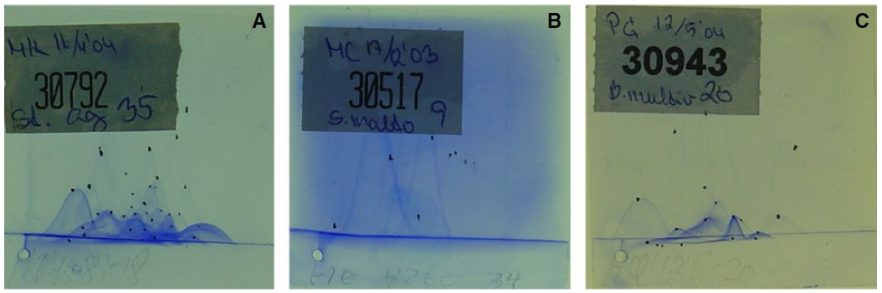


Fig. 3. (A) Crossed immunoelectrophoresis of Standard-Antigen (a sonicate of 17 differer groups) runs against serum from a CF patient with chronic *P. aeruginosa* lung infection. T

Table 2. Diagnostic use of three different anti-pseudomonas antibody methods (antibodies ir *Pseudomonas aeruginosa* biofilm infection in Scandinavian cystic fibrosis patients (17)

	Crossed immune-electrophoresis (St-Ag was used) (%)	*Pseudomona.* (St-Ag was u
Specificity	89	83
Sensitivity	96	97
Positive predictive value	87	80

Høiby N, et al. APMIS 2017;125:339

In vitro assessment of antimicrobial activity against biofilms: which tecnique ?

Susceptibility testing and PK/PD in biofilms

The classic antibiotic susceptibility tests (AST) are carried out using planktonic cells, under aerobic atmosphere, and at neutral pH levels comparable to those measured in human serum.

These conditions are diametrically opposed to those which microorganisms face at the site of CF infection:

Factors	Conventional ASTs	at infected CF lung
growth mode	single (free-floating) cells	aggregated (can be adhered) cells
atmosphere	aerobic	low O_2 tension, anaerobic
pH	neutral	acidic

Results from conventional ASTs cannot be therefore used to predict the therapeutic success for biofilm infections:

- Minimal Inhibitory Concentration (MIC)
- PK/PD parameters predicting therapeutic success

There is, therefore, increasing need in the development of AST specific to biofilm-growing bacteria simulating physico-chemical conditions observed in CF lung

Diagnostic Microbiology and Infectious Disease 82 (2015) 318–325

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In vitro activity of colistin against biofilm by *Pseudomonas aerugi*

significantly improved under "cystic fibrosis–like"

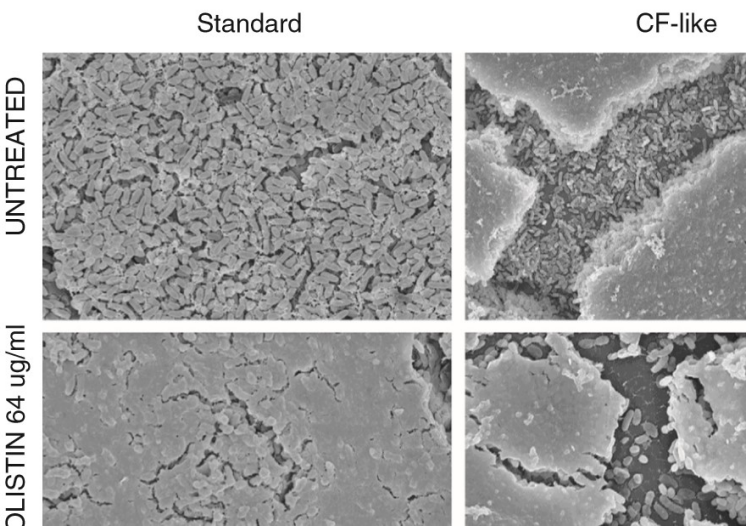

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Table 2
Antibacterial activity of colistin against planktc
from CF patients.

Strain	MIC (μg/mL)	
	Under the following conditions:	
	"Standard"	"C
Pa1	2	1
Pa2	4	**1**
Pa3	4	**0.5**
Pa4	4	**0.2**
Pa5	4	**0.5**
Pa6	4	**0.5**
Pa7	2	**0.5**
Pa9	4	**0.5**
Pa10	2	**0.2**
Pa16	2	**0.1**
Pa18	4	**0.5**
Pa21	4	**0.2**
MIC_{50}[a]	4	**0.5**
MIC_{90}[b]	4	**1**
MIC_{range}	2-4	0.1
MBC_{50}[c]		
MBC_{90}[d]		
MBC_{range}		
Mean MBC/MIC[e]	1.16	4.8

vels did not significant-
er absolute biofilm bio-
suggested by high SD
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erestimation of colistin

strains, comparatively
s summarized in Table 2.
d MIC_{90} values signifi-
.5 and 4 μg/mL, respec-
Pa1, all of strains tested
like" conditions.
sidered did not differ for
g/mL, for "CF-like" and
otient (MBC/MIC ratio)
endent on experimental
condition (mean MBC/
C/MIC: 4.8).

rmed by P. aeruginosa

Table 3
In vitro activity of colistin against 5-day-old bi
isolated from CF patients.

Strain	MBEC (μg/mL)	
	Under the following conditions:	
	"Standard"	"C
Pa1	256	
Pa2	128	
Pa3	64	
Pa4	512	
Pa5	256	
Pa6	>1024	
Pa7	1024	
Pa9	64	
Pa10	1024	
Pa16	32	
Pa18	256	
Pa21	1024	
$MBEC_{50}$[a]	256	
$MBEC_{90}$[b]	1024	

- colistin activity against both planktonic and biofilm *P. aeruginosa* cells is significantly increased in CF-like conditions (acidified and anaerobic)
- **It is needed to adequately "rethink" the current protocols used for assessing antibiotic efficacy**, by considering experimental conditions simulating the actual physicochemical and microbiological characteristics of the CF lung ecosystem

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REVIEW ARTICLE

Critical review on biofilm methods

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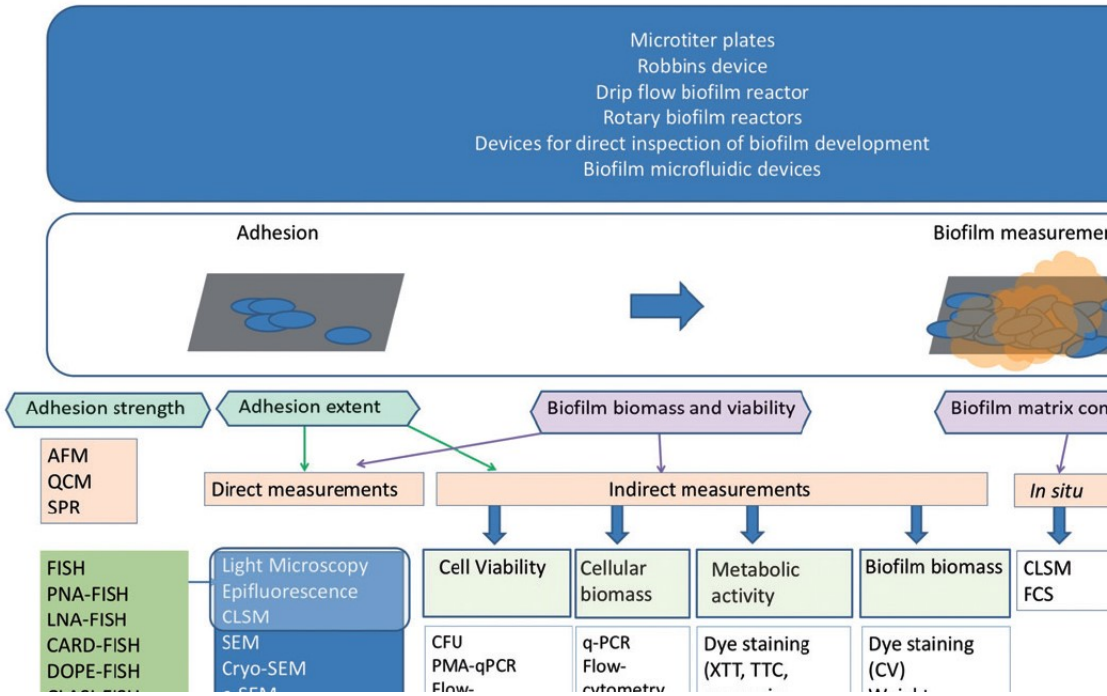


Several *in vitro* biofilm models were described, basically classifiable in two types:

- open and dynamic
- closed and static

Commonly employed models for biofilm investigation

open and dynamic | closed and static

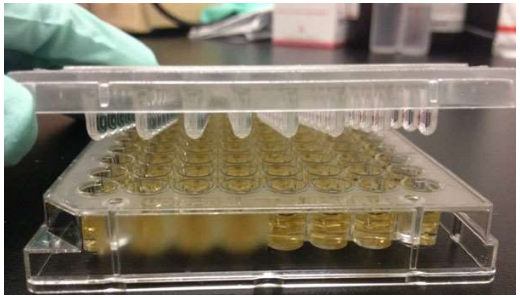
Biofilm model	Method	Nutrient availability	Potential applications and relevance
Rotating disc reactor (annular reactor) (ATSM E2196 – approved 2002)	This test method is used for growing a reproducible *P. aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions	Open system Dynamic Continuous flow	Rotating disc reactors are designed for laboratory evaluations of biocide efficacy, biofilm removal, and performance of anti-fouling materials. Example is to model a toilet bowl [49]. It is important to note that the rotating disk and CDC reactor were not originally designed to study medically relevant biofilms
Drip flow reactor (ATSM E2647 – approved 2008)	This test method is to grow, sample, and analyze a *P. aeruginosa* biofilm under low fluid shear and close to the air/liquid interface	Open system Dynamic Batch or continuous flow	DFR are employed for growing biofilms for direct *in situ* visualization. The DFR can model environments such as food-processing conveyor belts, catheters, and the oral cavity [50,51]
CDC biofilm reactor (ATSM E2562 – approved 2007)	This test method is used for growing *P. aeruginosa* biofilm under moderate-to-high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one particular environment	Open system Dynamic Batch or continuous flow	Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure [52], and assessing the effect of antimicrobial agents on the biofilm (Note there is a large body of literature on how researchers are using the CDC, DFR and MBEC for various research applications)
MBEC assay /microtiter plates (ASTM E2799 – approved 2011)	This test method specifies the operational parameters required to grow and treat a P. biofilm in a high-throughput screening assay	Closed system Low shear (the reactor sits on a shaker) Batch	MBEC assay allows rapid throughput of multiple samples of anti-biofilm therapeutics such as antibiotics, antiseptics, compounds, and peptides [53]
Single tube disinfection (ATSM 2871 – approved 2013)	Standard test method for evaluating disinfectant efficacy against *P. aeruginosa* biofilm grown in the CDC biofilm reactor using the single tube method	The single tube method is only an efficacy test. Biocides are tested in a batch system, with no mixing at room temperature	This test was originally designed to determine the efficacy of liquid biocides against biofilm (bleach, quats, hydrogen peroxide blends, etc.). Although it has been optimized using biofilm grown in the CDC reactor, the original intent was that the biofilm could originate from any biofilm reactor, as long as the appropriate controls were carried along

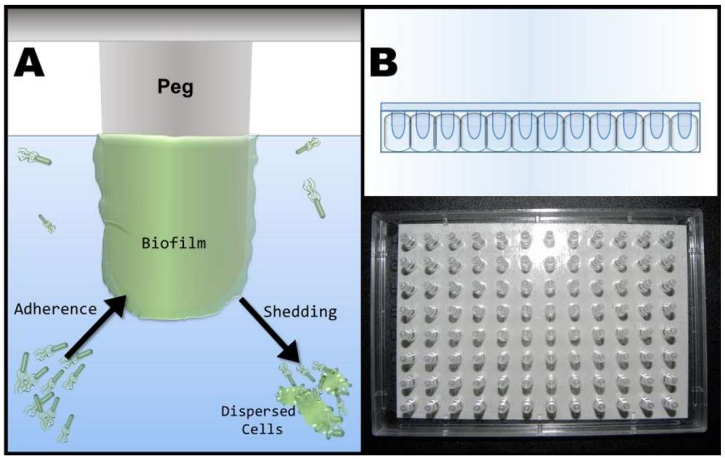
Malone M, et al. Expert Rev Anti Infect Ther. 2017;15(2):147-156

Susceptibility testing and PK/PD in biofilms
In vitro models

Microtiter plate-based Calgary device

- closed and static: medium is not added or removed during biofilm formation
- easy to perform and compatible with laboratory routine work, useful for high throughput screening
- biofilm biomass was assessed spectrophotometrically (OD) or by cell viable count
- microscopic analysis can be performed





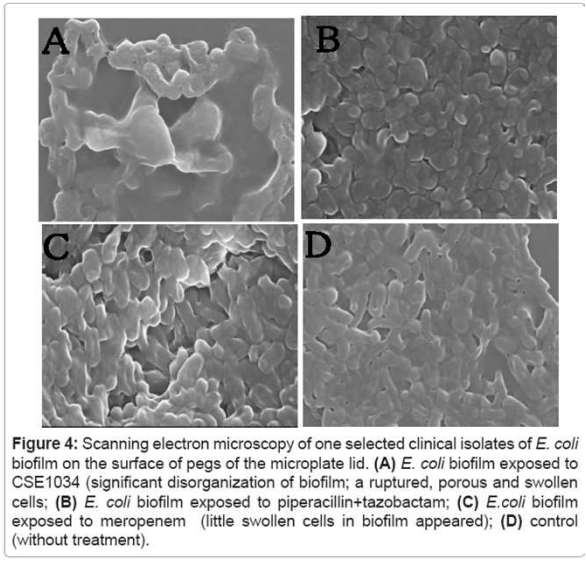


Figure 4: Scanning electron microscopy of one selected clinical isolates of *E. coli* biofilm on the surface of pegs of the microplate lid. **(A)** *E. coli* biofilm exposed to CSE1034 (significant disorganization of biofilm; a ruptured, porous and swollen cells; **(B)** *E. coli* biofilm exposed to piperacillin+tazobactam; **(C)** *E.coli* biofilm exposed to meropenem (little swollen cells in biofilm appeared); **(D)** control (without treatment).

In vitro endpoints to assess antimicrobial activity against biofilms

Susceptibility testing and PK/PD in biofilms
PD parameters to quantify antimicrobial activity against biofilms

- **Minimal Biofilm Inhibitory Concentration (MBIC)**: the lowest concentration causing a OD_{650} reduction ≤ 10%
- **Biofilm Bactericidal Concentration (BBC)**: the lowest concentration killing 99.9% of biofilm cells
- **Minimal Biofilm Eradication Concentration (MBEC)**: the lowest concentration eradicating biofilm (100% killing)
- **Biofilm Prevention Concentration (BPC)**: the lowest concentration preventing biofillm formation; useful in CF patients during early stage of *P. aeruginosa* colonization

Breakpoints are not yet available for biofilm growing bacteria.

However, comparison of planktonic and biofilm PD parameters gives us important information on the anti-biofilm effects of antibiotics:

- MIC vs MBIC
- MBC vs (BBC or MBEC)

Antibiotic activity against *P. aeruginosa* biofilm
In vitro studies

- **Azithromycin** is particulary effective, although long-term therapy selects for hypermutable resistant strains.[21] Hyperexpression of MexCD-OprJ confers cross-resistance to others not antipseudomonal agents[22]
- **Ciprofloxacin**, active on biofilm in Calgary device, selects for resistant mutants in the flow cell model,[23] also at 2 mg/L concentration (mutant prevention concentration, ΔUC/MIC: 384)
- Other PK/PD studies[24-27] showed:
  - time-dependent killing of **β-lactams**
  - concentration- or dose-dependent killing for **ciprofloxacin**, **colistin** and **tobramycin**
  - site-dependent killing: metabolically active outer layers (**ciprofloxacin, beta-lactams, tobramycin**), or quiescent inner layers (**colistin**), providing a rationale for combined therapy[27]

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Standard versus biofilm antimicrobial susceptibilit

Implications for practice

- **There is insufficient evidence to recommend choosing antibiotics based on current biofilm AST rather than conventional AST in the treatment of *P. aeruginosa* pulmonary infections in CF people**. *In vitro* biofilm AST systems cannot currently predict better antibiotic choices for the treatment of CF pulmonary infections.

Implications for research

- Testing antimicrobials against bacterial biofilms in the laboratory may be more appropriate in the development of newer, more effective formulations of drugs, able to penetrate CF sputum and bacterial structures, which can then be tested in clinical trials.

Comparison: antibiotics chosen on the basis of standard antimicrobial susceptibility test

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No of particip (studie
	Assumed risk	Corresponding risk		
	Standard antimicrobial susceptibility testing	Biofilm antimicrobial susceptibility testing		
FEV_1 change from start of treatment (L) Follow-up: 14 days	The mean change in FEV_1 ranged across control groups from 0.12 L to 2.75 L.	The mean change in FEV_1 in the intervention groups was 0.04 L higher (0.08 L lower to 0.16 L higher).	NA	68 (2)
FEV_1 change from start of treatment (% predicted) Follow-up: 14 days	The mean (SD) change in FEV_1 in the control group was 9.62 (10.12)% predicted.	The mean change in FEV_1 in the intervention groups was 2.47% lower (9.29% lower to 4.34% higher).	NA	34 (1)
Adverse events: number of moderate adverse events. Follow-up: duration of antibiotic treatment (14 days)	129 per 1000	46 per 1000 (9 to 228)	RR 0.36 (0.07 to 1.77)	73 (2)
Sputum density: change in *P aeruginosa* sputum density (log_{10} CFU/g) Follow-up: 14 days	The mean change in sputum density ranged across control groups from -3.27 to -3.83 log_{10} CFU/g.	The mean change in sputum density in the intervention groups was 0.8 log_{10} CFU/g higher (0.59 log_{10} CFU/g lower to 2.18 log_{10} CFU/g higher).	NA	70 (2)
Quality of life:	The mean change in CFQ-R	The mean change in CFQ-R score in the	NA	38 (1)

Antibiotic activity against *P. aeruginosa* biofilm
Humanization of *in vitro* studies

Although all of the *in vitro* systems can be reproducibly used for testing the effects of antibiotics on biofilm, they fail to mimic both the complexity of the host environment and the pathogen-host interactions

- Need for deeper *in vitro* and *in vivo* studies to design antibiotic strategies based on AST of biofilm.
- Humanization of *in vitro* models trying to simulate physico-chemical and biological conditions observed at the site of infection:
  - seaweed **alginate-embedded** bioflms[28]
  - using a flow cell simulator, **meropenem** at 2 g (single bolus) killed young but not older (thicker) biofilm[29]
  - **artificial sputum medium** (ASM) and microaerophilic atmosphere to simulate CF environment[30,31]
  - **mixed/multispecies biofilm**[32-34]

In vivo endpoints to assess antimicrobial activity against biofilms

Endpoints of antimicrobial treatment of biofilm infections *in vivo*

- The general endpoint of acute infections (i.e. pneumonia, sepsis) is curative.
- In the case of biofilms, it is important to establish the final aim of the treatment. In CF patients:
  - with **intermittent colonization** of lungs, therapy should be aimed at **eradicating** the infection (planktonic or unstructured young biofilm)
  - once **chronic infection** is established, therapy is aimed at **suppression** (maintenance therapy) of infection with bacterial load reduction in order to maintain the lung function
- **Difficulty in diagnosing biofilm makes indirect criteria more used than microbiological ones**:
  - improvement in clinical symptoms and functional tests, decreased inflammatory response, imagistic improvement in lesions
- ***In vivo* animal models** have been widely used:[35,36]
  - reflect the ongoing battle between pathogens and host immune response, but are unable to mimic the long-term inflammatory response and substantial antibiotic treatment. In CF lung, this interplay can last up to 30 years, resulting in both phenotypic and genotypic bacterial variants[37]
  - the microbiological response (reduction in bacterial load) to therapy is used as endpoint

In vivo CF model

INFECTION AND IMMUNITY, June 2010, p. 2466–2476
0019-9567/10/$12.00 doi:10.1128/IAI.01391-09

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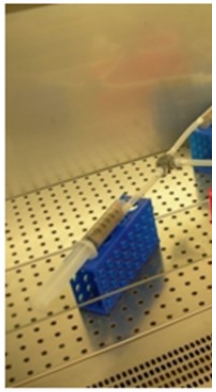
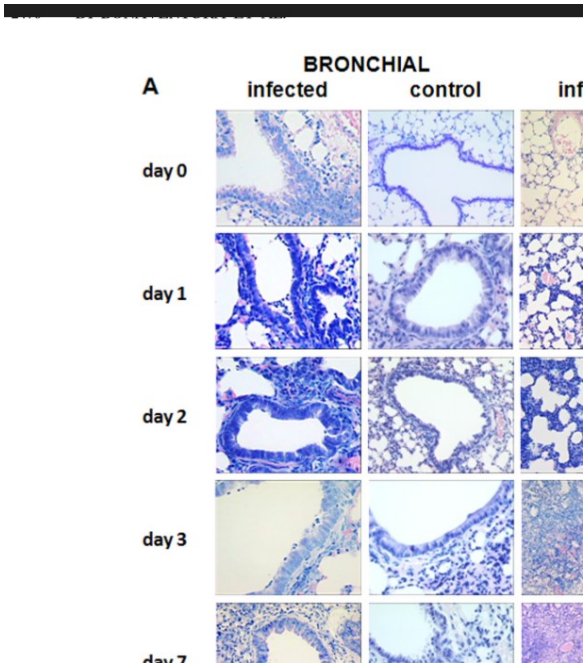
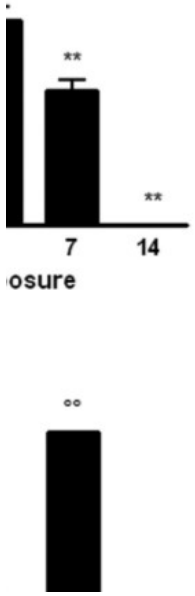


Role of Excessive Inflammatory Respons
Stenotrophomonas maltophilia Lung Infect
in DBA/2 Mice and Implications





LUNG INFECTION BY *STENOTRO*

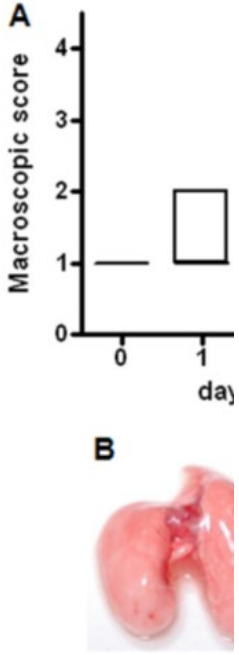


FIG. 5. Macroscopic path

Antibiotic strategies for combacting biofilm infections in CF patients

Antibiotic strategies against biofilm
High antibiotic concentrations through topical administration

- High local concentrations of antibiotic delivered by nebulization directly to the site of infection
- Treatment of choice in suppressive or maintenance therapy in CF patents
- Advantages:
  - **no side effects** (low serum levels); nebulized tobramycin: 1200 mg/L in sputum, only <1 mg/L in serum[38]
  - **decreases chance to develop antibiotic resistance**
  - **effective vs resistant strains** (concentration is well higher than MIC)
  - **improves pulmonary symptoms, reduces bacterial load in sputum, well tolerated**[39,40]

Table 2. Summary of current topical antibiotic treatment regimens according to th

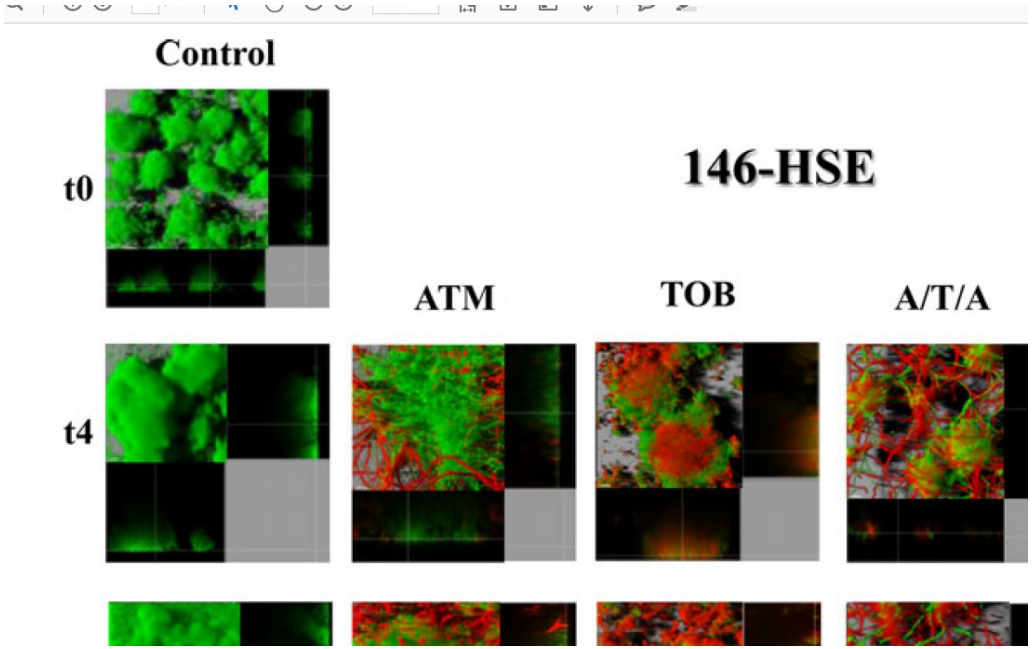
Biofilm site of infection	Antibiotic regimen	Duration
Lung infection in CF	0.5–2 MU colistin, twice daily	Continuo
	300 mg tobramycin, twice daily	28 days c cycles
	112 mg tobramycin dry powder, twice daily	On/off cy

Antibiotic strategies against biofilm
Combined antimicrobial therapies

- A combined therapy, especially in CF patients, is routinely used with the aim of preventing or delaying the onset of resistance[41]
- In the case of treatment of biofilm-related infections, combined therapies acquire an even more relevant dimension
- Rational approaches for establishing combination therapy:
  - biofilms exhibit different metabolic states: combination of agents active vs metabolically active layers (ciprofloxacin, tobramycin or the beta-lactams) with others (colistin) that preferentially kill biofilm cells with low metabolic activity:[27] **colistin + tobramycin**[42]
  - multiple combination bactericidal testing has been shown to help to choose combinations of antimicrobials with higher levels of *in vitro* bactericidal activity, especially in *P. aeruginosa*[43] and *Burkholderia cepacia* complex.[44] However, there is insufficient evidence to determine the advantage of choosing antibiotics based on combination AST vs conventional AST in the treatment of pulmonary exacerbations in CF patients with chronic *P. aeruginosa* infection.[45-47] Prospective large international and multicenter trials are needed.
  - promising combinations of inhaled antibiotics such as **clarithromycin + tobramycin** or **colistin + tobramycin** are still at a very early stage of development[48]

Antibiotic strategies against biofilm
Sequential antimicrobial therapies

- Another approach to prevent or delay the onset of resistance may be the use of **sequential treatments based on antagonistic interactions**.
- Treatment with aminoglycosides often involves the selection of mutants overexpressing MexXY-OprM efflux pump, related to the inactivation of MexAB-OprM.[49]
- Therefore, treatment with MexXY-OprM substrates (such as tobramycin) might theoretically lead to hypersusceptibility to MexAB-OprM substrates (such as aztreonam); **tobramycin followed by aztreonam** would entail a clinical benefit by improving the therapeutic efficacy and diminishing the selection of resistant mutants.
- It has been recently observed that sequential therapies with inhaled tobramycin and aztreonam were found to be superior to individual treatments.[50]

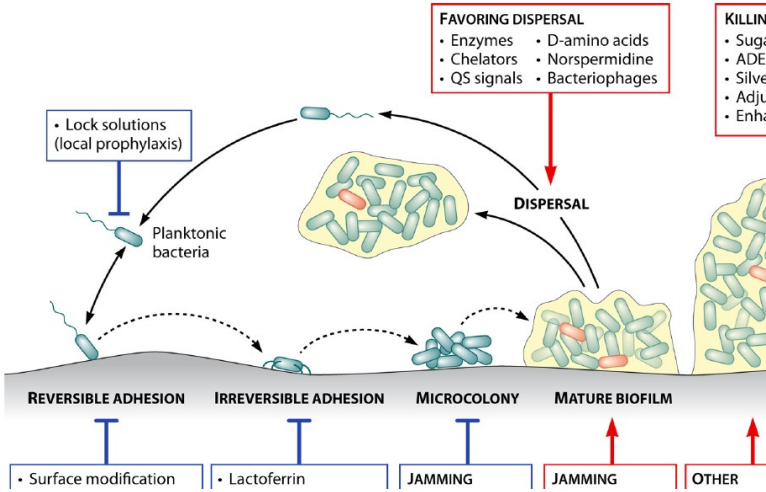


Biofilms by *P. aeruginosa* 146-HSE Liverpool epidemic strain (GFP-tagged) are treated with peak aztreonam (ATM, 700 mg/L) and tobramycin (TOB, 1000 mg/L), and stained with propidium iodide (red). Images obtained at: t0 (2-day-old biofilm), t4 (6-day-old biofilm, 4 days of treatment) and t6 (8-day-old biofilm, 6 days of treatment). A/T/A stands for the alternation of ATM/TOB/ATM and T/A/T for the alternation of TOB/ATM/TOB. (Rojo-Molinero et al., 2016)

New approaches for eradicating biofilms

New approaches for eradicating biofilms

- Several **therapeutic failures are still being observed**:
  - cure rates never reach 100%; treatment failure can reach 50%, depending on host and pathogen factors
  - prolonged antibiotic treatment is frequently required, leading to increased selective pressure and the risk of antibiotic resistance, medical cost and toxicity
- **Alternative therapeutic strategies, used alone or in combination with antibiotics** to increase the likelihood of biofilm eradication or to reduce the length of treatment, are therefore viewed as modern "holy grails". Among these:
  - antimicrobial peptides
  - natural compounds (i.e. secondary metabolites of lichens)
  - phages
  - enzymes degrading EPS
  - increase in O_2 tension
  - QS inhibitors



Conclusions

Over the last two decades both scientific and medical communities had a greater awareness of the role of biofilms in human health and disease. However, **we are not further along in the battle against biofilm-associated infections**:

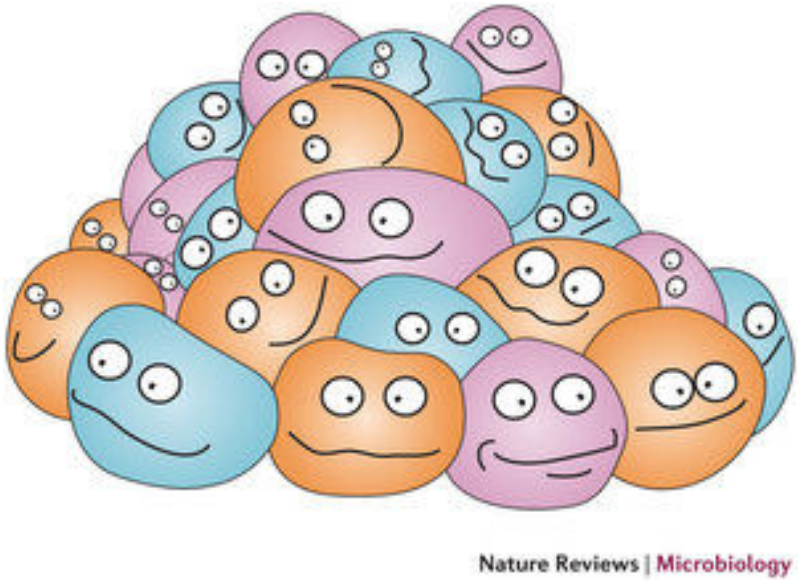
- minimal data correlating *in vitro* results to clinical outcomes
- clinicians find difficult to understand how *in vitro* methods translate to something of clinical relevance

When clinicians come across a new drug, the regulations on the wording of the claim/documentation is focused on curing or preventing infection. Biofilm does not become part of the discussion. To let the biofilm be included as part of the clinician's decision-making in terms of infection management we need:

- a **standardized laboratory diagnosis of biofilm-related infection**: clinicians need to start asking if the patient has a chronic biofilm or an acute infection
- a **standardized ad simple-to-use biofilm assay highly predictive of *in vivo* outcomes**; current *in vitro* tests are not predicting how the antibiotic will perform clinically
- an **appropriate outcome**, so clinicians understand the "effectiveness" of a drug, whether biofilm was reduced (if so, by how much?) or even eradicated; importantly, any reductions or killing of a biofilm should be associated with a reduction of infective symptoms and improved patient outcome

Due to the multi-factorial nature of biofilm recalcitrance to antibiotics, a **combination of the different strategies for improvement of the effect of antibiotics and of the immune system on biofilms is probably necessary**.

Thank you all for your attention !



References

1. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G. 1994. Biofilms, the customized microniche. J. Bacteriol. 176: 2137-2142.
2. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. 54: 49-79.
3. Wei Q, Ma LZ. 2013. Biofilm matrix and its regulation in Pseudomonas aeruginosa. Int. J. Mol. Sci. 14: 20983-21005.
4. Sauer, K. (2003) The genomics and proteomics of biofilm formation. Genome Biol. 4, 219
5. Klausen, M. et al. (2003) Involvement of bacterial migration in the development of complex multicellular structures in Pseudomonas aeruginosa biofilms. Mol. Microbiol. 50, 61–68
6. Sauer, K. et al. (2002) Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J. Bacteriol. 184, 1140–1154
7. Costerton, J.W. et al. (1999) Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322
8. Semmler, A.B. et al. (1999) A re-examination of twitching motility in Pseudomonas aeruginosa. Microbiology 145, 2863–2873
9. Klausen, M. et al. (2003) Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol. Microbiol. 48, 1511–1524
10. Kaplan, J.B. et al. (2003) Detachment of Actinobacillus actinomycetemcomitans biofilm cells by an endogenous betahexosaminidase activity. J. Bacteriol. 185, 4693–4698
11. Webb, J.S. et al. (2003) Cell death in Pseudomonas aeruginosa biofilm development. J. Bacteriol. 185, 4585–4592
12. Rice, S.A. et al. (2009) The biofilm life cycle and virulence of Pseudomonas aeruginosa are dependent on a filamentous prophage. ISME J. 3, 271–282
13. Isles, A. et al. (1984) Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J. Pediatr. 104, 206–210
14. Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8: 881-890.
15. Singh R, Paul D, Jain RK. 2006. Biofilms: implications in bioremediation. Trends Microbiol. 14: 389-397.
16. Høiby N. Pseudomonas aeruginosa Infection in cystic fibrosis. Diagnostic and prognostic significance of P.aeruginosa precipitins determined by means of crossed immunoelectrophoresis. A survey. Acta Pathol Microbiol Immunol Scand 1977;262(Suppl):1–96.
17. Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, et al. 2002. Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev. Cell 3: 593-603.
18. Jensen, P.O. et al. (2010) The immune system vs. Pseudomonas aeruginosa biofilms. FEMS Immunol. Med. Microbiol. 59, 292–305
19. Yoon SS, Hasset DJ. 2004. Chronic Pseudomonas aeruginosa infection in cystic fibrosis airway disease: metabolic changes that unravel novel drug targets. Expert Rev. AntiInfect. Ther. 2: 611-623.
20. Tolker-Nielsen T. 2014. Pseudomonas aeruginosa biofilm infections: from molecular biofilm biology to new treatment possibilities. APMIS Suppl. 122: 1-51.
21. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. J Clin Microbiol 2004;42:1915–22.
22. Mulet X, Macia MD, Mena A, Juan C, Perez JL, Oliver A. Azithromycin in Pseudomonas aeruginosa biofilms: bactericidal activity and selection of nfxB mutants. Antimicrob Agents Chemother 2009;53:1552–60.
23. Macia MD, Perez JL, Molin S, Oliver A. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of Pseudomonas aeruginosa biofilm treatment. Antimicrob Agents Chemother 2011;55:5230–7.
24. Hengzhuang W, Wu H, Ciofu O, Song Z, Hoiby N. Pharmacokinetics/pharmacodynamics of colistin and imipenem on mucoid and nonmucoid Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2011;55:4469–74.
25. Hengzhuang W, Hoiby N, Ciofu O. Pharmacokinetics and pharmacodynamics of antibiotics in biofilm infections of pseudomonas aeruginosa in vitro and in vivo. Methods Mol Biol 2014;1147:239–54.
26. Hengzhuang W, Ciofu O, Yang L, Wu H, Song Z, Oliver A, et al. High beta-lactamase levels change the pharmacodynamics of beta-lactam antibiotics in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2013;57:196–204.
27. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in P. aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol Microbiol 2008;68:223-40.
28. Cao B, Christophersen L, Thomsen K, Sonderholm M, Bjarnsholt T, Jensen PO, et al. Antibiotic penetration and bacterial killing in a Pseudomonas aeruginosa biofilm model. J Antimicrob Chemother 2015;70:2057–63.
29. Haagensen JA, Verotta D, Huang L, Spormann A, Yang K. New in vitro model to study the effect of human simulated antibiotic concentrations on bacterial biofilms. Antimicrob Agents Chemother 2015;59:4074–81.
30. Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. Use of artificial sputum medium to test antibiotic efficacy against Pseudomonas aeruginosa in conditions more relevant to the cystic fibrosis lung. J Vis Exp 2012;5:e3857.
31. Kolpen M, Appeldorff CF, Brandt S, Mousavi N, Kragh KN, Aydogan S, et al. Increased bactericidal activity of colistin on Pseudomonas aeruginosa biofilms in anaerobic conditions. Pathog Dis 2016;74:ftv086.
32. Brackman G, Garcia-Fernandez MJ, et al. Dressings loaded with cyclodextrin-hamamelitannin complexes increase Staphylococcus aureus susceptibility toward antibiotics both in single as well as in mixed biofilm communities. Macromol Biosci 2016;16:859–69.
33. van der Waal SV, de Almeida J, Krom BP, de Soet JJ, Crielaard W. Diffusion of antimicrobials in multispecies biofilms evaluated in a new biofilm model. Int Endod J. 2016; doi: 10.1111/iej.12634
34. Pompilio A, Crocetta V, De Nicola S, Verginelli F, Fiscarelli E, Di Bonaventura G. Cooperative pathogenicity in cystic fibrosis: Stenotrophomonas maltophilia modulates Pseudomonas aeruginosa virulence in mixed biofilm. Front Microbiol. 2015 Sep 16;6:951.
35. Di Bonaventura G, Pompilio A, Zappacosta R, Petrucci F, Fiscarelli E, et al. Role of excessive inflammatory response to Stenotrophomonas maltophilia lung infection in DBA/2 mice and implications for cystic fibrosis. Infect Immun. 2010 Jun;78(6):2466-76.
36. Bragonzi A. Murine models of acute and chronic lung infection with cystic fibrosis pathogens. Int J Med Microbiol. 2010 Dec;300(8):584-93.
37. Bjarnsholt, T. et al. (2010) Quorum sensing and virulence of Pseudomonas aeruginosa during lung infection of cystic fibrosis patients. PLoS ONE 5, e10115
38. Yu Q, Griffin EF, Moreau-Marquis S, et al. In vitro evaluation of tobramycin and aztreonam versus Pseudomonas aeruginosa biofilms on cystic fibrosis-derived human airway epithelial cells. J Antimicrob Chemother 2012;67:2673–81.
39. Retsch-Bogart GZ, Quittner AL, Gibson RL, Oermann CM, McCoy KS, Montgomery AB, et al. Efficacy and safety of inhaled aztreonam lysine for airway pseudomonas in cystic fibrosis. Chest 2009;135:1223–32.
40. Stass H1, Delesen H, Nagelschmitz J, Staab D. Safety and pharmacokinetics of ciprofloxacin dry powder for inhalation in cystic fibrosis: a phase I, randomized, single-dose, dose-escalation study. J Aerosol Med Pulm Drug Deliv 2015;28:106–15.

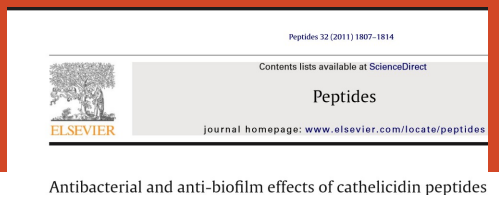
References

41. Doring G, Flume P, Heijerman H, Elborn JS. Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros 2012;11:461–79.
42. Herrmann G, Yang L, Wu H, Song Z, Wang H, Hoiby N, et al. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm Pseudomonas aeruginosa. J Infect Dis 2010 Nov;202:1585–92.
43. Lang BJ, Aaron SD, Ferris W, et al. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of Pseudomonas aeruginosa. Am J Resp Crit Care Med 2000;162:2241–5.
44. Aaron SD, Ferris W, Henry DA, Speert DP, Macdonald NE. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am J Respir Crit Care Med 2000;161:1206–12.
45. Aaron SD, Vandemheen KL, Ferris W, et al. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. Lancet 2005;366:463–71.
46. Haja Mydin H, Corris PA, et al. Targeted antibiotic prophylaxis for lung transplantation in cystic fibrosis patients colonised with Pseudomonas aeruginosa using multiple combination bactericidal testing. J Transplant 2012;2012:135738.
47. Waters V, Ratjen F. Combination antimicrobial susceptibility testing for acute exacerbations in chronic infection of Pseudomonas aeruginosa in cystic fibrosis. Cochrane Database Syst Rev 2015;11:CD006961.
48. Antoniu S. Novel inhaled combined antibiotic formulations in the treatment of Pseudomonas aeruginosa airways infections in cystic fibrosis. Expert Rev Anti Infect Ther 2015;13:897–905.
49. Vettoretti L, Plesiat P, Muller C, El Garch F, Phan G, Attree I, et al. Efflux unbalance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. Antimicrob Agents Chemother 2009;53:1987–97.
50. Rojo-Molinero E, Macià MD, Rubio R, et al. Sequential treatment of biofilms with aztreonam and tobramycin is a novel strategy for combating P. aeruginosa chronic respiratory infections. Antimicrob Agents Chemother 2016;60:2912–22.

Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev. 2014 Sep;78(3):510-43.
Ciofu O, Rojo-Molinero E, Macia MD, Oliver A. Antibiotic treatment of biofilm infections. APMIS 2017; 125:304–319.
Malone M, Goeres DM, Gosbell I, Vickery K, Jensen S, Stoodley P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. Expert Rev Anti Infect Ther. 2017 Feb;15(2):147-156.
Høiby N, Bjarnsholt T, Moser C, Jensen PØ, Kolpen M, Qvist T, Aanaes K, Pressler T, Skov M, Ciofu O. Diagnosis of biofilm infections in cystic fibrosis patients. APMIS. 2017 Apr;125(4):339-343.
Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, Jensen PØ, Høiby N. The in vivo biofilm. Trends Microbiol. 2013 Sep;21(9):466-74.

New approaches for eradicating biofilms
Antimicrobial peptides

- Anti-Microbial Peptides (AMPs) are under the spotlight as a promising class of antimicrobials for development as novel antibiotics.[1]
- Naturally occurring molecules of the innate immune system of animals with important roles in host defense.[2,3]
- Most AMPs have a wide spectrum of activity (comprising MDR pathogens), a relatively good selectivity toward bacteria, and a rapid mechanism of action, often based on the lysis/permeabilization of microbial membranes. This mode of action, in which no specific molecular targets are involved, is associated with a low frequency for selection of resistant strains.
- **We have shown that some bovine alpha-helical AMPs[4] have a potent and rapid *in vitro* bactericidal and anti-biofilm activity against *P. aeruginosa* and *S. maltophilia* strains from CF patients.[5,6]**
- **However, poor *in vivo* activity due to enzymatic degradation and cytotoxic effect remain to be solved.[4-6]**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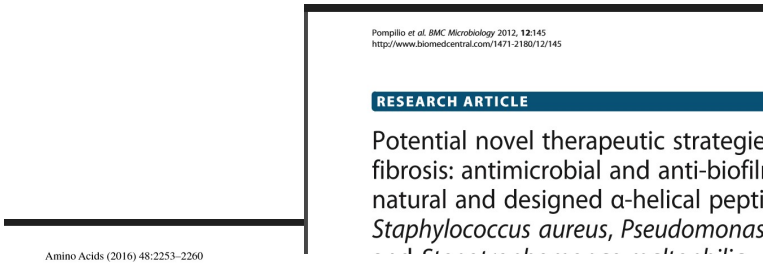
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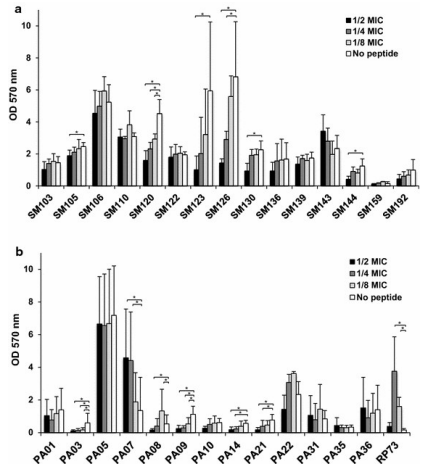

New approaches for eradicating biofilms
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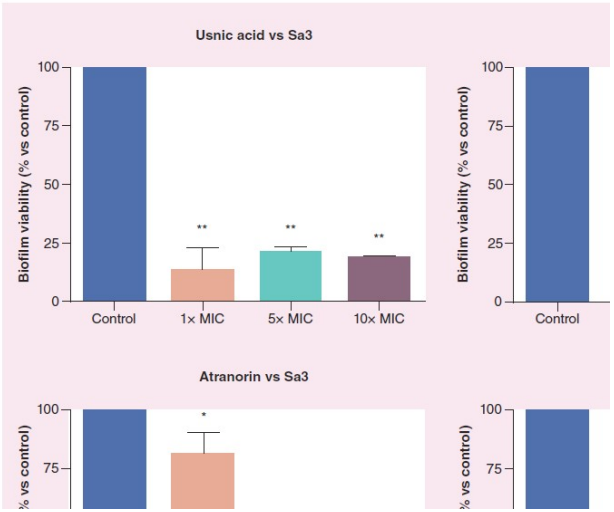
at concentrations that are significantly lower

antimicrobial agents and pathogen erad



RESEARCH ARTICLE
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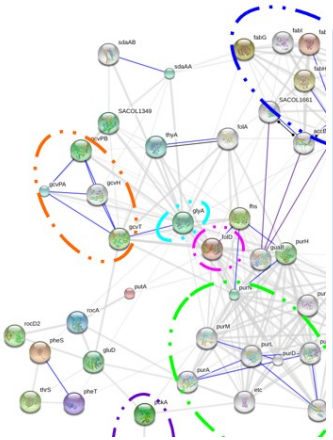
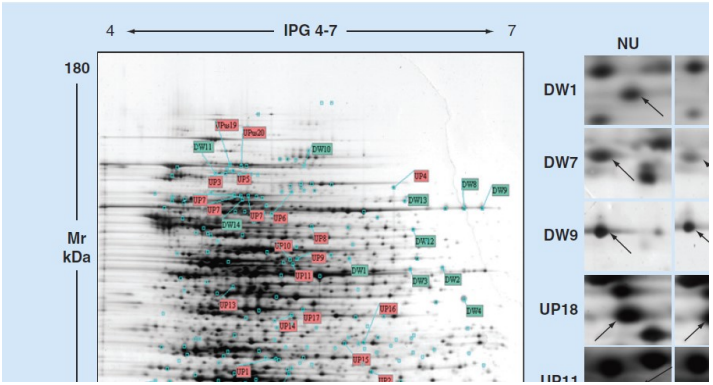
Evaluation of antibacterial and antibiofilm mechanisms by usnic acid against methicillin-resistant *Staphylococcus aureus*





Usnic acid shows relevant activity against *S. aureus* CF strains, causing damage in peptidoglycan synthesis. It also affects both adhesion and biofilm formation (due to impaired adhesion to the host matrix binding proteins), reduces viability of preformed biofilms, and decreases virulence (lipase and thermonuclease expression)

New approaches for eradicating biofilms
Extremely low-frequency magnetic field

PRELIMINARY COMMUNICATION

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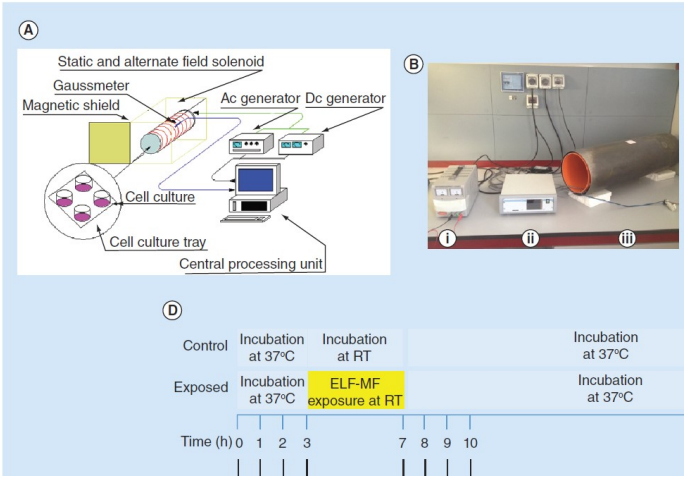
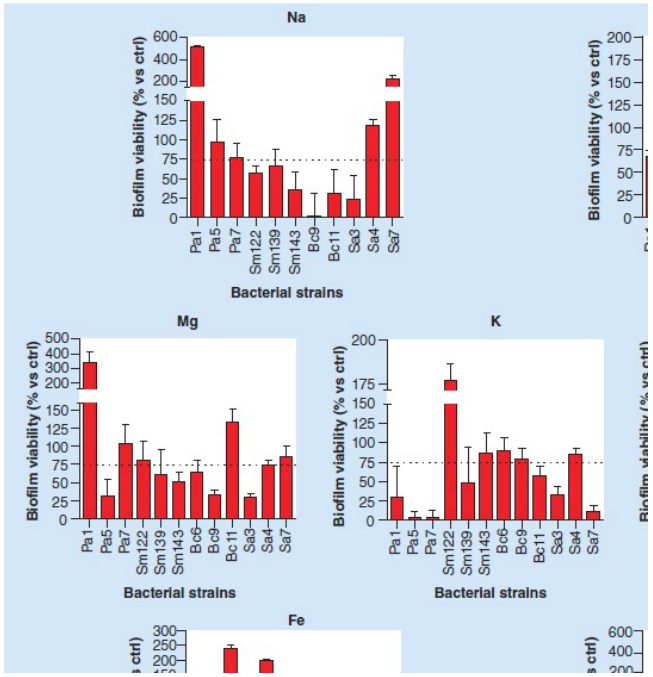
Exposure to extremely low-frequency magnetic field affects biofilm formati by cystic fibrosis pathoge



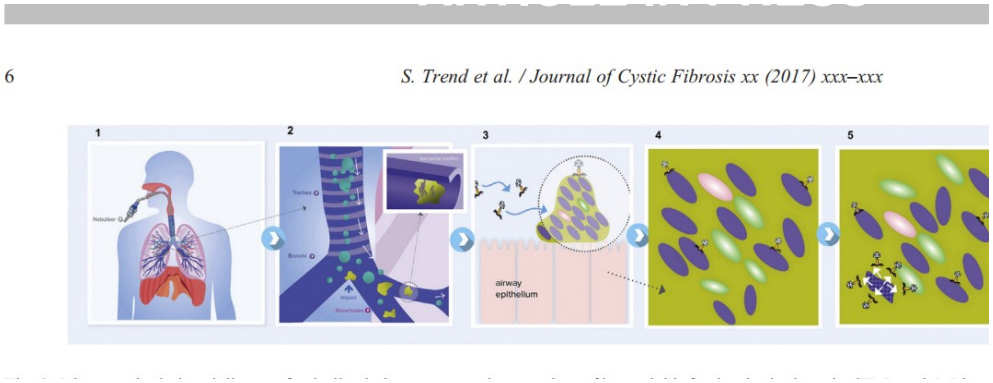
PRELIMINARY COMMUNICATION Di Bonaventura, Pompilio, Crocetta *et al*

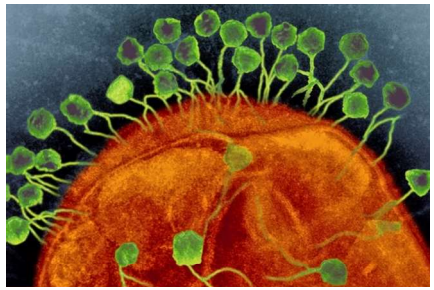


Exposure to ELF-MF significantly decreases biofilm formation by CF pathogens, probably not depending on a bactericidal effect but rather to reduced bacterial adherence to substratum secondary to altered permeability of the ionic channels of cell membrane

New approaches for eradicating biofilms
Phage therapy

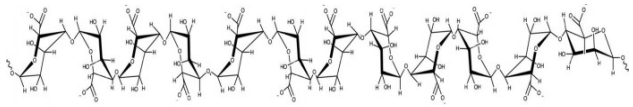
- A range of phages have potential medicinal use due to broad *in vitro* antibacterial activity against CF pathogens[1]
- Cocktails of phages or phages + antibiotics have also proposed to kill preformed biofilms
- **We have recently isolated new phages able to disperse *P. aeruginosa* biofilm; better results when administered in combination with antibiotics (tobramycin, meropenem)**
- Potential causes of failure of phage therapy in the CF airway:
  - the inability of the phage to make physical contact with the target bacterial cells
  - bacterial strains having or developing resistance to a phage through mutation and natural selection
  - in a chronic infection, phages bind to numerous dead cells in which they cannot replicate





New approaches for eradicating biofilms
EPS components degradation

- In chronic murine lung infection an **oligomer of alginate** which destabilizes the alginate matrix (OligoG; AlgiPharma, Sandvika, Norway) improves the effect of the immune system and antibiotics on *P. aeruginosa* biofilm.[151-155] OligoG is developed as a dry powder for inhalation, and a solution for nebulization, as an orphan drug product for treating CF patients. A phase 2b clinical trial in CF patients is in progress.
- The matrix entraps bacterially produced enzymes such as β-lactamases. The hydrolyzation of β-lactam antibiotics by the biofilm matrix can change the PK/PD of the β-lactam antibiotics in biofilms from time- to dosage-dependent, meaning that both time of exposure and concentration of β-lactam are important for the effect on biofilms.[8] This can be bypassed by the **use of β-lactamase-stable β-lactam antibiotics (meropenem, imipenem)** or higher dosages for longer periods of time of **β-lactamase-unstable antibiotics (ceftazidime)**.
- **DNase I**, an enzyme degrading DNA, was efficiently used to dissolve biofilms from a broad range of bacteria, including *P. aeruginosa.*[400] Use of nebulized DNase seems to reduce the incidence of new infections in CF patients.
- Use of **bacteriophage-derived alginate lyase**, such as PT-6, depolymerizes *P. aeruginosa* alginate inducing biofilm dispersal.[362]



New approaches for eradicating biofilms
Increase in O_2 tension

- *In vivo* **low O_2 tension** at the CF infection site impairs the efficacy of ROS-dependent bactericidal antibiotics (i.e. ciprofloxacin) and decreases bacterial metabolism[15]
- **Increase in O_2 tension by hyperbaric oxygen treatment** (HBOT) (100%, 2.8 bar) enhances the efficacy of antibiotic treatment on both planktonic[142,143] and biofilm grown *P. aeruginosa* treated with **fluoroquinolones**[144]
- In addition, HBOT affects immune cell functions as the killing capacity can be improved by the production of reactive oxygen species during their oxidative burst[145]
- HBOT can be considered as an adjuvant both for the activity of bactericidal antibiotics and of the inflammatory cells, although extensive optimization of the HBOT treatment is required before proceeding to clinical trials
- For the same reason, bactericidal antibiotics not relying on ROS formation, such as colistin, can beneficially be used, probably in combination therapy, for example with fluoroquinolones for biofilm treatment:
  - **colistin + ciprofloxacin** has been successfully used in eradicating *P. aeruginosa* infection in CF patients[146]

New approaches for eradicating biofilms
Quorum-sensing inhibitors

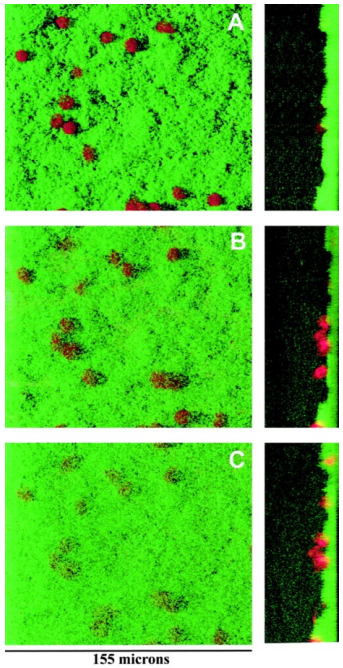
- The precise role of QS in biofilm formation in CF remains unclear. However, there is evidence suggesting that **QS plays a role in the viability of *P. aeruginosa* anaerobic biofilms**.[4]
- **QS signaling can be targeted to interfere with biofilm formation and also to trigger dispersal of a biofilm**.
- In *S. aureus*, the *agr* (accessory gene regulator) QS system is strongly expressed at the moment of dispersion. Artificial stimulation of this system, through adjunction of autoinducing peptide (AIP), leads to *S. aureus* biofilm dispersal.[5] *In vivo* murine models also helped to reveal the effect of RIP (a quorum sensing inhibitor) in combination with teicoplanin against methicillin-resistant *S. aureus*.[6]
- In *P. aeruginosa*, the short-chain fatty acid implicated in bacterium-bacterium communication (cis-2-decenoic acid) is able to induce dispersal in a wide range of Gram-positive as well as Gram-negative bacteria.[7]

- Silver-coated nanoparticles have also proposed to kill preformed biofilms.

New approaches for eradicating biofilms
Increasing immune system efficacy

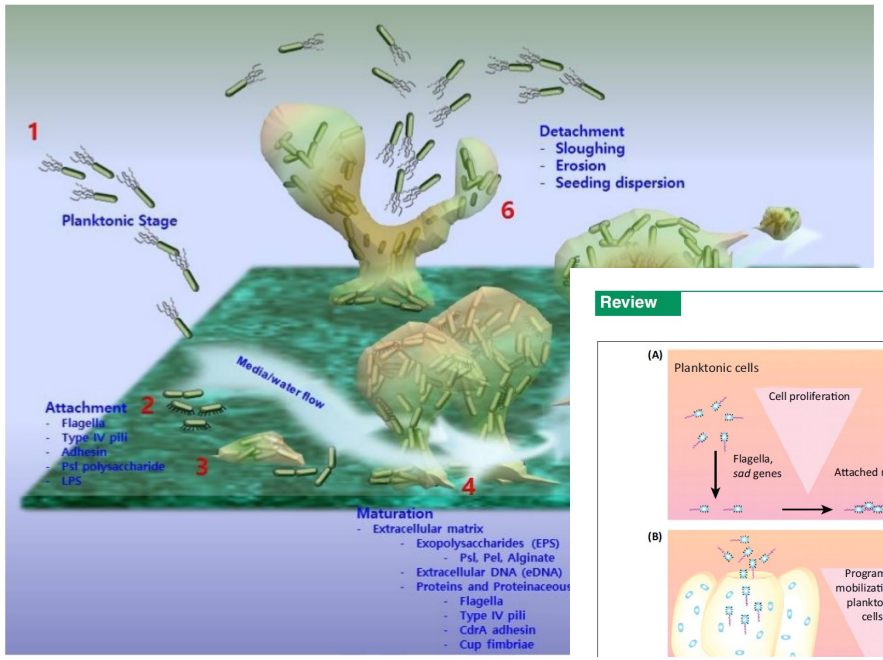
As the *in vivo* eradication of the biofilms is an interplay between antibiotics and the immune system, **increasing the eradication potential of the immune system** is an important part of the treatment of biofilm infections.

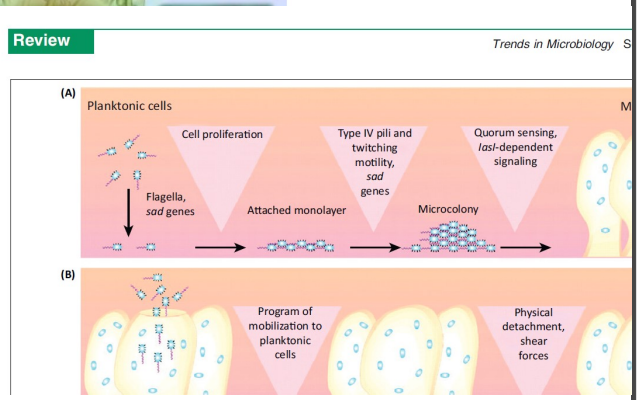
- After interaction with *P. aeruginosa* biofilms, neutrophils become phagocytically engorged, partially degranulated, immobilized, and rounded. This also causes increased O_2 consumption due to both bacterial respiration and escape response and the neutrophil respiratory burst, with low concentration of H_2O_2 . Thus, host defense becomes compromised as biofilm bacteria escape while neutrophils remain immobilized with a diminished oxidative potential.
- It has recently been shown that activated leukocytes can actively phagocytose biofilm bacteria. VISTO IN CF PATIENTS ?



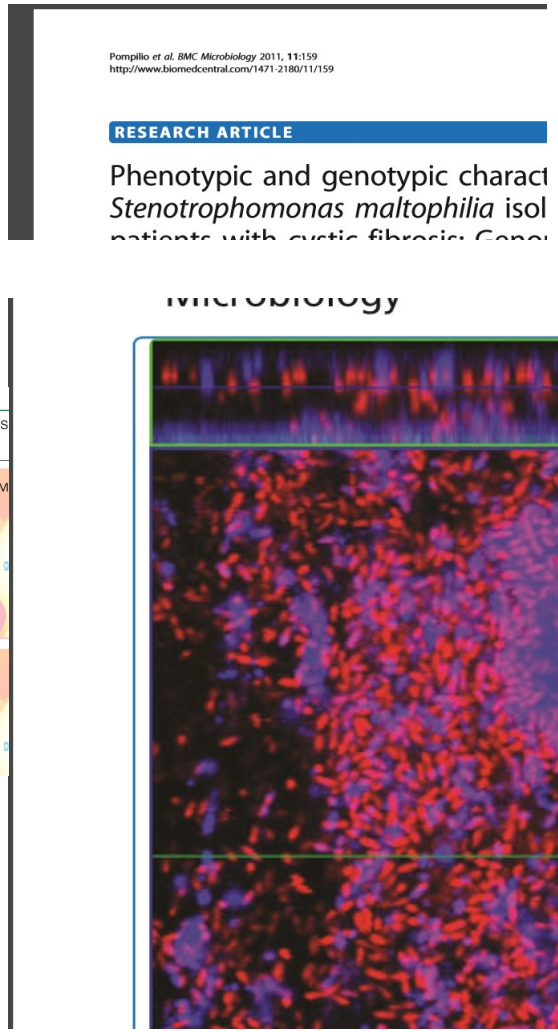
Time course of settling of neutrophil on *P. aeruginosa* PAO1 (pMF230) biofilms. *A*,5 min following neutrophil addition, *B*, 15 min, and *C*, 60 min. The confocal transverse sections projections are in adjacent panels. *Jesaitis et al, J Immunol 2003.*

Biofilm formation





Diagrammatic representation of the developmental stages of *P. aeruginosa* biofilm. (1) the planktonic stage, (2) attachment of bacteria to a surface, (3) production of the extracellular matrix, (4) maturation of biofilm structures, (5) spatial differentiation, and (6) biofilm dispersal (*Lee et al, J Microb Biotechnol, 2017*).



Diagnosis of biofilm-based infection

16–20) (Tables 1–2) (Fig. 3A). Likewise, the (Figs 3B, C, D and 4) (21

Table 1. Current laboratory methods for diagnosis *Pseudomonas aeruginosa* and other bacteri
sputum or mucus from paranasal sinuses in CF

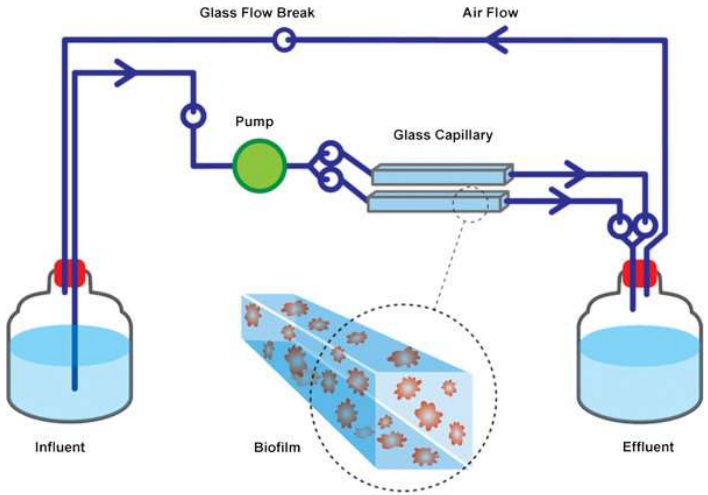
Microscopy:	Light microscopy of Gram-stained smears, the biofilms are small aggre the matrix is dominated by alginate, and it may take several minutes There is abundance of polymorphonuclear leukocytes around the bio (Fig. 1A–C, Fig. 2A–D) (15)
	FISH microscopy of smears, the biofilms are small aggregates (4–100 µ minutes to find a biofilm (Fig. 1D). There is abundance of polymorpl around the biofilms. The signal of the FISH probe is dependent on th in each bacterial cell and dormant or slow growing bacteria may ther fluorescence (28)
	The polysaccharide matrix of the biofilms can be stained by Alcian blu
Growth of mucoid colonies of *P. aeruginosa* (Fig. 5) (5, 18)	

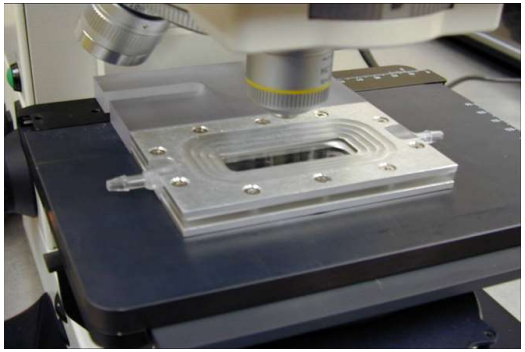
Høiby N, et al. APMIS 2017;125:339

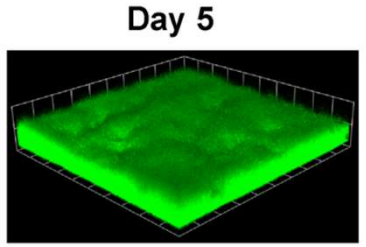
Susceptibility testing and PK/PD in biofilms
In vitro models

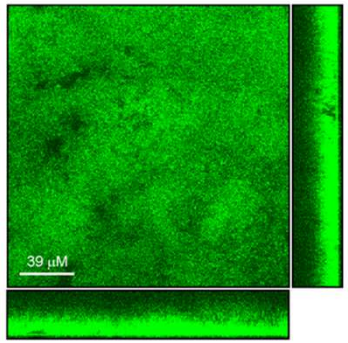
<u>Flow cell, bioreactor</u>:

- opened and dynamic: during biofilm formation, fresh medium is added and spent medium was removed thus causing turbolence (shear forces)
- real-time non-destructive CLSM in situ analysis can be performed to assess viability (fluorescent tag) and to perform structural analysis by dedicated software (COMSTAT)









Mechanisms of biofilm's tolerance to antibiotics

Table 1. Mechanisms of tolerance to antibiotics in biofilms (adapted after 26)

Antibiotics	(Physical) Biofilm matrix-related tolerance	Physiological tolerance (low metabolic activity and slow growth)	Adaptive
Beta-lactam	• Diffusion partially impaired (27)	• No activity on non-dividing cells (28)	• Inductic hydroly: can imp • Subinhil alginate • Upregul response
Quinolones	• No impact (31)	• Impaired activity in anaerobic conditions (20, 32)	• Upregul response • Upregul pump in
Aminoglycosides	• Diffusion impaired by alginate (34) • eDNA creates cation-limited conditions and induces LPS modification and impaired uptake of antibiotics (5)	• Impaired activity on non-dividing cells (35)	• Upregul • Subinhil biofilm • *ndv*-depe periplas this con • Upregul pump in
Antimicrobial	• eDNA creates cation-limited		• Specific:

Ciofu O, et al. APMIS 2017;125(4):304-319