Biofilm Antimicrobial Susceptibility Testing: Where Are We and Where Could We Be Going?

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Summary

Our knowledge about fundamental aspects of biofilm biology, including the mechanisms behind the reduced antimicrobial susceptibility of biofilms, has increased drastically over the last decades. However, this knowledge has so far not been translated into major changes in clinical practice. While the biofilm concept is increasingly on the radar of clinical microbiologists, physicians and healthcare professionals in general, the standardized tools to study biofilms in the clinical microbiology laboratory are still lacking; one area in which this is particularly obvious is that of antimicrobial susceptibility testing (AST). It is generally accepted that the biofilm lifestyle has a tremendous impact on antibiotic susceptibility, yet AST is typically still carried out with planktonic cells. On top of that, the microenvironment at the site of infection is an important driver for microbial physiology and hence susceptibility, but this is poorly reflected in current AST methods. The goal of this review is to provide an overview of the state-of-the-art concerning biofilm AST and highlight the knowledge gaps in this area. Subsequently, potential ways to improve biofilm-based AST will be discussed. Finally, bottlenecks currently preventing the use of biofilm AST in clinical practice, as well as the steps needed to get past these bottlenecks, will be discussed.

INTRODUCTION

Microbial biofilms are communities of one or more microorganisms (bacteria and/or fungi) embedded in an extracellular polymeric matrix (produced at least partially by the microorganisms themselves); biofilms can be surface-attached or occur as suspended aggregates (1-3). Although cells in surface-attached biofilms and suspended aggregates show the same phenotype (1), the molecular mechanisms underlying their formation are not necessarily identical (4). In line with previous work, microbial aggregates will be defined as biofilms in this text, regardless of whether they are attached to a biotic or abiotic surface (1).

Microbial biofilms are present in virtually every ecological niche on Earth and it has been estimated that 40-80% of all microbial cells are biofilm-associated (5). An estimated 65-80% of all infections is considered to be biofilm-related (6, 7) and although it is not always completely clear what criteria are used to define an infection as biofilm-related, there is no doubt they have a considerable impact on morbidity, mortality, and healthcare-related costs (8). Biofilms can be found in many types of infections and while typically associated with chronic infections, recent data point to a role for biofilms in acute infections as well (9, 10). Many biofilms are associated with the use of indwelling medical devices, including (but not limited to) cardiovascular implants, intravascular devices, orthopedic implants (mainly knees and hips), urinary catheters, endotracheal tubes, breast implants, contact lenses, dental implants and intrauterine devices (8, 11-16). Risk factors for developing a chronic-device related infection include immunomodulatory therapy, diabetes, smoking, and renal disease, suggesting that a compromised innate immune response increases the risk for developing these infections (17). However, not all biofilm infections are related to the use of medical devices, and examples of native tissue biofilms include these identified in respiratory tract infections (e.g. in patients with cystic fibrosis (CF) and chronic rhinosinusitis), chronic otitis media, native valve endocarditis, the oral cavity and chronically infected wounds (14, 18-22).

While our knowledge about fundamental aspects of microbial biofilms (including knowledge concerning the mechanisms behind their reduced antimicrobial susceptibility) has increased tremendously over the past decades (1, 13, 23-26), the translation of this increased knowledge about biofilm biology to clinical practice is lagging behind. That does not mean no progress was made: for example guidelines for improved diagnosis of biofilm-associated infections have been published (27, 28) and at least for prosthetic joint infections 'biofilm-active' antibiotics (e.g. rifampicin, ciprofloxacin) have been identified (29-31). However, biofilm-based susceptibility testing, i.e. antimicrobial susceptibility testing (AST) using biofilm-grown bacteria to select the antibiotic(s) to treat a biofilm-related infection, has not yet found its way to the clinical microbiology laboratory, although proposed technologies to do so have been around for over two decades (32). In the present review I outline the state-of-the-art concerning biofilm AST, highlight the knowledge gaps, and propose solutions to improve biofilm-based AST. In addition, I will discuss what will likely be needed for these biofilm AST methods to be implemented in the clinical microbiology laboratory.

CURRENT APPROACHES FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

Conventional approaches

In most cases (empirical therapy being the notable exception), the selection of antimicrobial therapy is made based on the susceptibility profile of the infecting organism, as determined using phenotypic tests in which susceptibility is quantified by measuring the effect of the antibiotic on bacterial or fungal growth, using broth microdilution or gradient strip-based methods. Values obtained in these tests (i.e. minimal inhibitory concentrations, MICs) are then compared to breakpoints established for specific dosing regimens by international organizations like EUCAST and CLSI (33, 34): if the MIC is below the breakpoint, the organism is considered susceptible to the antibiotic, and therapy with this antibiotic is predicted to be successful. Alternatively, susceptibility can be assessed using disk diffusion assays in which susceptibility is quantified based on the size of the inhibition zone (35, 36). While there are automated systems for phenotypic susceptibility testing (37), the majority of these also rely on growth of the bacterium and as a consequence it typically takes 1-2 days to complete the test for rapidly growing microorganisms, and even more time is required for fastidious, slow-growing microorganisms.

Genomic detection of resistance mechanisms

A potential solution for the latter problem is to move beyond phenotypic (growth-based) susceptibility testing, and to use bacterial whole genome sequences (WGS) to infer antimicrobial susceptibility (38-42). However, most WGS-based approaches focus on finding known resistance mechanisms and while they are successful in that, identifying (combinations of) mutations in one or more genes not previously associated with reduced susceptibility, and incorporating these in a prediction algorithm, remains a major challenge (43). In addition, information derived from WGS cannot predict expression patterns of genes involved in antimicrobial susceptibility in specific conditions (44). Indeed, the specific conditions in a biofilm and at the infection site lead to distinct gene expression profiles that are different from those observed *in vitro* (45-47), complicating the prediction of biofilm susceptibility based on WGS. For example, several biofilm-specific efflux systems have been described (48, 49) as well as the biofilm-specific synthesis of cyclic-β-1,3-glucans that sequester antibiotics (50) and these mechanisms would be difficult to pick up with WGS alone.

Alternative Methods for Susceptibility Testing

An alternative approach potentially yielding faster results relies on mass spectrometry (more specifically on matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MALDI-TOF MS). With MALDI-TOF MS, a spectrum can be obtained from a microbial sample that can be used for rapid and accurate identification to the species level (51, 52) but also to predict antimicrobial susceptibility (53-55). Discrimination between susceptible and resistant isolates can be made based on presence/absence or change in intensity of certain peaks in the MALDI-TOF spectrum (56, 57). More recently, advanced machine learning algorithms have been used to predict antimicrobial susceptibility of various pathogens based on MALDI-TOF profiles (58-60).

Heat is a by-product of the majority of biological processes; the amount produced is directly related to growth and the heat production rate is related to the metabolic fluxes; using microcalorimetric devices, the energy released during metabolic processes in microorganisms can be measured (61). Microcalorimetry has two major advantages, (i) it is label-free and can be applied in virtually all conditions (e.g. also in turbid media containing blood) and (ii) it allows real-time measurements. Microcalorimetry has been used to determine antimicrobial susceptibility in different organisms and the results obtained so far look are overall in agreement with results obtained with conventional susceptibility tests (62-68).

Alternative culture-based approaches for AST are also being developed. An example of such an approach is the AtbFinder system, in which a medium is used that supports growth of many different bacteria (TGV medium) (69, 70). The system is based on direct plating of clinical specimens on TGV agar, with or without antibiotics added at a concentration that can be achieved at the infection site; the approach claims to also consider polymicrobial interactions influencing antimicrobial susceptibility. Case studies have suggested this approach leads to selection of antibiotics with better efficacy for treating nosocomial pneumonia (71) and chronic relapsing urinary tract infections (72). A recently-published clinical trial in which the AtbFinder system was used in the context of respiratory tract infections in CF patients (35 patients, of which 33 were chronically colonized with *Pseudomonas aeruginosa*) suggests that antibiotics selected with AtbFinder lead to clearance of *P. aeruginosa*, a decrease in the number of pulmonary exacerbations, and an increase in lung function (73).

Finally, various microscopy-based approaches for AST have been developed (74-77). For example the Accelerate Pheno system uses tracking of the size, shape, and division rate of growing cells exposed to antibiotics, to estimate susceptibility (74, 75); in a clinical trial use of this system led to faster changes in antibiotic therapy for bloodstream infections caused by Gram-negative bacteria (78).

However, despite the promising results obtained with some of the alternative AST methods discussed above, additional validation will be required prior to their routine clinical use.

Shortcoming of Current Approaches

There is frequently a poor correlation between results obtained with *in vitro* susceptibility tests and the effect *in vivo*, for example in respiratory tract infections in patients with CF (79-81). Indeed, both pharmacodynamic parameters (determining the relationship between the concentration of the antibiotic at the site of action, and its physiological effects) and pharmacokinetic parameters (determining the relationship between the concentration of the antibiotic in body fluids and tissues, and time) are crucial for the activity of antibiotics *in vivo* (82-84). However, the behavior of microorganisms *in vitro* can be very different from that observed *in vivo*. An important factor contributing to failure of antimicrobial therapy is that *in vivo* microorganisms form biofilms that show reduced susceptibility towards antimicrobial agents (23, 25). Biofilm cells are phenotypically very different from planktonic cells and the microenvironment in these surface-attached or suspended biofilms (including gradients of O_2, nutrients and waste products) (85, 86), leads to an altered metabolism linked to reduced susceptibility (24). In addition, the spatial heterogeneity of biofilms may support diversification, i.e. the development of subpopulations with varying degrees of susceptibility, within a patient (87-90). The presence of such subpopulations leads to intrasample diversity in antibiotic susceptibility of isolates and raises questions about the validity of sampling procedures and the common practice of performing susceptibility testing on a limited number of isolates (91, 92). It is worth pointing out that this is not only the case for respiratory tract infections in CF patients, as adaptation and diversification (also in terms of antimicrobial susceptibility) are also observed in other diseases, including non-CF bronchiectasis and urinary tract infections (93-96). Finally, interactions between different microorganisms during (chronic) infections (97-102), as well as interactions between pathogens and the host (103, 104) play an important role in antimicrobial susceptibility, but are difficult to mimick *in vitro.*

BIOFILM-BASED ANTIMICROBIAL SUSCEPTIBILITY TESTING

Pharmacodynamic Parameters for the Assessment of Antimicrobial Activity in Biofilms

While the MIC and minimal bactericidal concentration (MBC, defined as the lowest concentration that kills all planktonic bacteria) are well-established parameters to assess antimicrobial activity and predict the success of a treatment, no such standardized parameters are available for biofilm susceptibility testing. Several parameters, including minimal biofilm inhibitory concentration (MBIC), biofilm inhibitory concentration (BIC), minimal biofilm eradication concentration (MBEC), biofilm prevention concentration (BPC), minimum biofilm bactericidal concentration (MBBC), minimum antibiotic concentration for killing (MCK) and biofilm tolerance factor (BTF) have been introduced as measures of biofilm susceptibility (105-111). However, their exact definition frequently varies between different studies and may also depend on the method used to quantify biofilms (e.g. plate counts, crystal violet staining, resazurin-based viability staining) (112, 113) (Table 1). On top of this lack of unambiguously defined pharmacodynamic parameters, there is also an overall lack of standardization in biofilm research that makes comparison between different studies difficult (114-116). Finally, no biofilm-specific breakpoints have been defined yet, complicating the interpretation and clinical use of the above-mentioned parameters.

Tools for Biofilm-based Antimicrobial Susceptibility Testing

While most studies on biofilm susceptibility use microtiter plate (MTP) based systems, in principle any biofilm model system can be used to determine biofilm susceptibility (12, 117-121). Nevertheless, specific methods for biofilm susceptibility testing have been developed and the most well-known in this context is the MBEC Assay Kit, also known as the Calgary Biofilm Device (32, 107). In this MTP based assay, biofilms are formed on plastic pegs (uncoated or coated) that are attached to the lid of a 96-well MTP and are immersed in a liquid; subsequently, the established biofilms are transferred to a new 96-well plate for AST (122). Examples of recently described advanced model systems for biofilm susceptibility testing include a microfluidic platform with an integrated sensor (the BiofilmChip) (123), an *ex vivo* CF lung model comprised of pig bronchiolar tissue and synthetic CF sputum (124), the BioFlux system (125, 126) and dissolvable alginate hydrogel-based biofilm microreactors (127). Other innovative models for biofilm AST were recently reviewed (128).

An important part of biofilm-based AST is the quantification of the number of (remaining) viable and/or culturable cells in treated and untreated biofilms. Quantification can be done using detached/dispersed cells, either immediately (i.e. plating of detached cells and counting CFUs after a suitably long incubation time) or after a re-growth phase. In the latter case, the presence or absence of growth can be measured (spectrophotometrically or by plating) or the length of the lag phase can be used to quantify the number of viable cells (129). Alternatively, quantification can be done directly on the biofilm, using for example ATP measurements, crystal violet staining, resazurin-based viability staining, microscopy, electrical impedance, or molecular methods (12, 123, 130-134). A detailed description of biofilm quantification approaches is outside the scope of the present review but it is important to reiterate that different quantification approaches often measure very different things (e.g. measuring optical density after regrowth does not allow to determine the log reduction in CFU, crystal violet stains more than only living cells etc), and that minor modifications to procedures may lead to different outcomes, as documented for example with crystal violet staining (115, 135). Crystal violet staining of surface-attached biofilms is arguable the most used technique, but due to its limitations, it is insufficient as the only method to measure biofilm reduction and it is recommended that results obtained with crystal violet staining are confirmed using other approaches (e.g. CFU counts, microscopy). In addition, in many studies, important characteristics like repeatability (i.e. the ability to obtain the same results when performing multiple tests in the same laboratory), reproducibility (i.e. the ability to obtain the same results when performing multiple tests across multiple laboratories) and responsiveness (i.e. the ability to differentiate between different concentrations of the treatment) (116, 136) are not investigated. A thorough assessment of these parameters is of course crucial prior to any clinical implementation. Examples of biofilm-based antimicrobial susceptibility test for which this was done include the MBEC biofilm disinfectant efficacy test (137) and several MTP based approaches (115).

Is There an Association Between Biofilm Formation and Antimicrobial Susceptibility?

If there would be an association between the biofilm formation *in vitro* (i.e. can an organism form a biofilm in a certain model system? how much biofilm is formed in a certain period of time?) and antimicrobial susceptibility (i.e. the MIC value), the capability and extent of biofilm formation could be used to predict susceptibility. Below I present a selection of the many studies in which this question has been addressed, organized per taxonomic group in order to facilitate comparisons between studies.

***Staphylococcus* spp.** Biofilm formation was associated with amikacin resistance in a collection of 49 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, but not with susceptibility to 15 other antibiotics (138). In a collection of 300 *S. aureus* isolates, no associations could be detected between methicillin-resistance and biofilm formation, while resistance to erythromycin, clindamycin and rifampin was associated with increased biofilm formation (139). In a collection of 111 staphylococci from prosthetic joint infections, no association was found between MBEC/MIC ratios and biofilm formation for *S. aureus*, while for *S. epidermidis* increased biofilm resistance (i.e. high MBEC/MIC ratio) to several antibiotics was observed in strong biofilm-producers (140). No significant differences were observed between the biofilm-forming capacity of methicillin-susceptible and methicillin-resistant *Staphylococcus* spp. isolates, or between isolates susceptible or resistant to most other tested antibiotics (total of 229 isolates investigated) (141). The exception was rifampicin: on average rifampicin-resistant strains formed significantly more biofilm than susceptible strains (141) (Fig. 1A). In a collection of 70 staphylococci from prosthetic joint infections, MBEC/MIC ratios for ciprofloxacin (but not for seven other antibiotics tested) were significantly higher for 'strong biofilm producers' than for 'non/weak producers' (142).

***Acinetobacter baumannii*.** In a collection of 271 *A. baumannii* isolates, non-multidrug-resistant (MDR) *A. baumannii* isolates tended to form stronger biofilms than MDR and extensively drug-resistant (XDR) strains. For 20/21 antibiotics tested (polymyxin being the exception), susceptible isolates were stronger biofilm formers than intermediate and resistant ones (143). However, in a study with 207 *A. baumannii* isolates, susceptible and less-susceptible strains were found to be equally capable of biofilm formation (144). Likewise, in a collection of 309 *A. baumannii* isolates, no difference was observed between MDR and non-MDR isolates in terms of their biofilm-forming capacity (145).

***Escherichia coli* and *Klebsiella pneumoniae*.** In a meta-analysis of the link between biofilm formation and antibiotic resistance in uropathogenic *E. coli* (17 studies included), 14 studies showed a positive association between biofilm formation and antibiotic resistance, two studies did not show any association and a single study reported a negative association between biofilm production and antibiotic resistance (146). Two studies addressed this question in *K. pneumoniae*. In a first study (120 isolates), XDR strains showed a higher ability to form biofilms than MDR and susceptible strains (147). In a second study with 100 *K. pneumoniae* isolates, ciprofloxacin-susceptible isolates formed stronger biofilms than resistant isolates; such a difference was however not observed for other antibiotics (148).

***Pseudomonas aeruginosa*.** Increased biofilm formation (as well as reduced motility) was observed in MDR/XDR high-risk *P. aeruginosa* clones (ST-111, ST-175, and ST-235) (149). However, in a collection of 302 *P. aeruginosa* isolates, the distribution of isolates with different biofilm-forming capacities did not differ among the MDR and non-MDR groups (150). In contrast, in a study with 66 isolates (of which 40 were MDR), an inverse association between resistance and biofilm formation was observed, with more biofilm formation in isolates categorized as non-MDR (151). Finally, a meta-analysis (20 eligible studies published between 2000 and 2019, on isolates recovered in Iran) found that overall biofilm formation was higher in MDR *P. aeruginosa*, although a significant association between biofilm formation and antibiotic resistance was only observed in 10 studies (50%) (152). The above-mentioned studies suggest that the interaction between antimicrobial resistance mechanisms and biofilm formation in *P. aeruginosa* is complex. For example, inactivation of the negative regulator NfxB leads to overexpression of the MexCD-OprJ efflux pump but also to impaired constitutive AmpC overexpression and consequently to decreased periplasmic β-lactamase activity (important for β-lactam resistance). While this leads to increased susceptibility to β-lactam antibiotics in planktonic cells, AmpC secreted by *nfxB* mutants still protects biofilm cells, probably due to the accumulation of AmpC in the biofilm matrix (153).

Discussion. The studies mentioned above clearly indicate that the question whether there is an association between biofilm formation and antimicrobial susceptibility is difficult to answer, with conclusions differing between different studies, even within the same taxonomic group. However, closer inspection reveals that the setup of many studies is suboptimal in terms of including a sufficiently diverse and large collection of isolates, the biofilm model system and quantification approach used, as well as analysis and interpretation of data. In many cases the biomass of surface-attached biofilms is indirectly quantified (e.g. by using crystal violet) and the values obtained are compared to that of a reference strain and/or arbitrary cut-offs. For example, in one study biofilms yielding optical density (OD) read-outs (at 550 nm, OD_{550nm}) after crystal violet staining that were higher than that of the negative control, but lower than that of a particular reference strain were designated as 'weak biofilm formers', while those with OD_{550nm} values higher than that of the reference strain were considered 'strong biofilm formers' (143). In another study the mean of blank-corrected OD values was used to group isolates into the categories 'nonproducer' (OD < 0.120), 'weak producer' (0.120 < OD < 0.240) and 'strong producer' (OD > 0.240) (140). While these approaches may work well within a single study, they will likely be difficult to reproduce between different laboratories and the biological relevance of the (seemingly arbitrary) cut-offs established is unclear. In addition, biofilm susceptibility is often defined based on the MIC of a particular antibiotic for a given isolate, and as discussed in more detail below, using breakpoints established for planktonic cells to categorize biofilms as 'susceptible' or 'resistant' may lead to misleading results. Finally, the *post hoc ergo propter hoc* assumption (after this, therefore because of this) is frequently made in studies in which a link between biofilm formation and antimicrobial susceptibility is observed, but we need to be careful to accept such an assumption. Biofilm formation and antimicrobial susceptibility (of planktonic and biofilm cells) are influenced by many factors, including stochastic events (e.g. stochastic formation of dormant persister cells) (154), variability in microbial populations (e.g. occurrence of heteroresistance in populations containing subpopulations of cells with lower susceptibility than the majority of the population) (155, 156) and the microenvironment (*in vitro* as well as *in vivo* at the site of infection, e.g. presence of certain nutrients) (26, 157, 158) and it may very well be that there simply is no mechanistic link between biofilm formation and planktonic susceptibility.

Can Biofilm Susceptibility Be Predicted Based on the MIC?

The question whether planktonic susceptibility can be used to predict biofilm susceptibility is an important one, because if MIC values, determined according to highly standardized EUCAST or CLSI procedures, would be a good proxy for biofilm susceptibility, dedicated biofilm AST would not be needed. Although planktonic and biofilm susceptibility parameter values for the same strain/antibiotic combinations have been determined in many studies, direct comparisons are again difficult due to differences in methodology and/or the lack of reporting susceptibility data for individual isolates. Below I focus on a selected set of studies that addressed this question for *P. aeruginosa* clinical isolates.

Moskowitz *et al*. compared susceptibility of planktonic cultures (MIC, determined according to CLSI guidelines) and biofilms (BIC, using the Calgary Biofilm Device) for 94 *P. aeruginosa* isolates towards 12 antibiotics (105). BICs were substantially higher than MICs for doxycycline and most of the β-lactam antibiotics investigated (aztreonam, ceftazidime, piperacillin-tazobactam and ticarcillin-clavulanate), while BICs of gentamicin and meropenem were only somewhat higher than the corresponding MICs, and BICs and MICs were fairly similar for amikacin, tobramycin and ciprofloxacin. Azithromycin showed fairly low BICs, although *P. aeruginosa* is considered as resistant in standard susceptibility testing. In a study with 57 non-mucoid *P. aeruginosa* isolates, planktonic (MIC) and biofilm (BPC, BIC) susceptibilities were determined for levofloxacin, ciprofloxacin, imipenem, ceftazidime, tobramycin, colistin and azithromycin (106). Some antibiotics showed median BPCs that were in the same range as MICs (fluoroquinolones, tobramycin, colistin), while others (ceftazidime, imipenem) had BPCs that were much higher than MICs. The former antibiotics also had relatively low BICs, indicating they may have activity against established biofilms. In a study with 133 *P. aeruginosa* isolates, marked differences between MIC and ‘biofilm active score’ (BAS) values (the latter determined based on microscopic assessment of the fraction of living cells after treatment) were observed for aztreonam and tobramycin (159). For 19.4% and 30.0% of the isolates that are resistant towards aztreonam and tobramycin, respectively, when grown planktonically, the biofilm biomass (as evaluated microscopically) was reduced with 50–75%. *Vice versa,* 63.6% of the aztreonam-sensitive and 66.2% of the tobramycin-sensitive isolates were non-responsive when grown as a biofilm. Using MIC, minimum antibiotic concentrations for killing (MCK, the concentration that resulted in a certain reduction in number of CFU of biofilm-grown cells) and the biofilm tolerance factor (BTF, the ratio of MCK and the MIC) (Table 1) as parameters for susceptibility to tobramycin, ciprofloxacin and colistin, Thöming & Häussler (110) observed that in a large (n=352) collection of clinical *P. aeruginosa* isolates, biofilm susceptibility values showed a wide distribution, even among isolates for which MIC values were similar; in addition, among isolates with a similar MCK value a wide spread in MIC values was observed (110). In a recent study, BPC values of tobramycin, ciprofloxacin or colistin (obtained with a resazurin-based viability staining on *P. aeruginosa* biofilms formed in a synthetic CF sputum medium) were at least four-fold higher than the MIC values (160) (Fig. 1B). However, BPC/MIC ratios were antibiotic-dependent, with BPC/MIC ratios for colistin being significantly higher than those for ciprofloxacin. Overall, a strong and significant rank correlation was observed between the MIC and the BPC for all antibiotics (i.e. strains showing higher MICs also show higher BPCs). Comparison of BPC with the MBC yielded a different picture. BPC values could be higher, equal or lower than the MBC and overall differences between BPC and MBC were smaller than differences between BPC and MIC. The BPC/MBC ratio was significantly smaller for ciprofloxacin than for colistin or tobramycin and while strong and significant correlations were observed between MBC and BPC for tobramycin and ciprofloxacin, this was not the case for colistin (160).

The selected studies discussed above suggest that while there may be an overall positive correlation between planktonic and biofilm susceptibility measurements, in many cases the reduced susceptibility observed in biofilms is independent of resistance in planktonic cultures. In addition, the relation between planktonic and biofilm susceptibility is antibiotic-dependent, and the impact of the biofilm model used and the stage in which the biofilms are tested on this relation is likely substantial (161-165). Finally, due to the lack of biofilm-specific antimicrobial susceptibility breakpoints, in many studies BPC, MBIC or MBEC values that are above the MIC are taken as evidence for 'biofilm resistance'. Considering the profound differences between planktonic cultures and biofilms, it seems however ill-advised to use breakpoints established for planktonic cells to categorize biofilms as 'susceptible' or 'resistant'.

Do Results of Biofilm-based Susceptibility Tests Correlate with Clinical Outcome?

While there are many *in vitro* studies in which planktonic and biofilm susceptibility towards different antibiotics are compared, there are few studies in which these data are linked to the clinical outcome of treatment with these particular antibiotics. Most of these pertain to prosthetic joint infections or respiratory tract infections in CF.

Prosthetic joint infections. In the context of prosthetic joint infections, biofilm-active antibiotics (defined as antibiotics that penetrate into the biofilm and are able to eradicate the bacteria in the biofilm) have been identified; these include rifampicin for staphylococci and ciprofloxacin for Gram-negative bacteria (31). A distinction is frequently made been 'difficult-to-treat' infections that are caused by pathogens resistant to these biofilm-active antibiotics, and prosthetic joint infections caused by susceptible organisms (29). Using a prospective cohort of patients (n=163) treated with a two-stage prosthesis exchange according to a standardized algorithm, Akgun *et al*. investigated whether the outcome of 'difficult-to-treat' prosthetic joint infections (n=30, 18.4%) is worse than that of other prosthetic joint infections (n=133, 81.6%) (166). While the infection-free survival rate at 2 years did not differ between both groups, hospital stay, prosthesis-free interval and duration of treatment were significantly longer in the 'difficult-to-treat' group than in the other group. This indicates that treatment with antibiotics that have activity against biofilms improves outcome, suggesting that knowing which antibiotic has an such an anti-biofilm activity could be clinically relevant. In a prospective cohort study with 131 patients with a prosthetic knee infection, outcome of treatment was compared between patients treated with biofilm-active antibiotics (n=55, 42%) or other antibiotics (n=76, 58%) (30). The infection-free survival after 1 year and 2 years was significantly higher for patients who received biofilm-active antibiotics and treatment with biofilm-active antibiotics was associated with lower pain intensity (30). In a group of 93 patients with infected spinal implants, treatment outcome was also compared between patients receiving biofilm-active antibiotics (n=30, 32%) and those who received no biofilm-active antibiotics (n=63, 68%). The infection-free survival differed significantly between both groups: for patients who received biofilm-active antibiotics it was 94% and 84% after 1 and 2 years, respectively, while it was only 57% and 49% for patients who received no biofilm-active antibiotics. In addition, patients receiving biofilm-active antimicrobial therapy reported lower intensity of postoperative pain (167). In a retrospective, observational, multicenter study involving 203 cases, treatment with biofilm-active antibiotics (rifampicin/fluoroquinolones) had a favorable impact on infections caused by staphylococci and Gram-negative bacteria. For example, the combination fluoroquinolone/rifampicin for staphylococcal infections significantly reduced implant failure (2% compared to 11% in the control group) (168). However, despite these observations, no association between MBEC values (for oxacillin, daptomycin, levofloxacin, rifampicin and levofloxacin/rifampicin combinations) and clinical outcome was observed in a study with 88 patients with a *S. aureus* prosthetic joint infection (169). This seems to contradict the evidence that the good *in vitro* anti-biofilm activity of antibiotic combinations containing rifampicin translates into high activity in animal prosthetic joint infection models and in patients suffering from biofilm-associated staphylococcal prosthetic joint infections (142, 170-176). It should be noted that the addition of rifampicin to the standard treatment did not lead to better outcomes in a recent clinical trial (177), although the setup of this trial was later criticized (31, 178). In two recent studies, MBEC/MIC ratios were determined for staphylococci recovered from prosthetic joint infections and linked to clinical outcome (140, 142). In both studies these ratios were lowest for rifampicin, again suggesting rifampicin has good antibiofilm activity *in vivo*. For 70 strains recovered from 49 patients with a first-time prosthetic joint infection (monomicrobial infection caused by staphylococci or polymicrobial infection caused by two different species of staphylococci), the oxacillin MBEC/MIC ratios were significantly higher in recurrent infections compared to resolved infections; no significant differences between the two patient groups were observed for MBEC/MIC ratios for other antibiotics (142). In a subsequent study (111 staphylococcal strains from 66 patients), the increased oxacillin MBEC/MIC ratios for *S. aureus* from unresolved prosthetic joint infections (median MBEC/MIC ratio of 1166 for isolates from unresolved infections vs. median MBEC/MIC ratio of 808 for isolates from resolved infections) was confirmed (140), suggesting that high relative MBEC values (compared to the MIC) are associated with poorer treatment outcome after a staphylococcal prosthetic joint infection. There are less data on the added value of using biofilm-active fluoroquinolones against prosthetic joint infections caused by Gram-negatives. In a study with 47 patients with acute prosthetic joint infections caused by a Gram-negative organism, treatment with a fluoroquinolone (when all the strains isolated were susceptible to this antibiotic) was associated with a good prognosis (179). In a study on 160 patients with an early prosthetic joint infection, treatment failed in 43 patients (26.9%) and the presence of a Gram-negative infection not treated with fluoroquinolones was identified as an independent predictor of therapy failure (180). Finally, in patients with prosthetic joint infections due to ciprofloxacin-susceptible Gram-negatives, the success rate of treatment was 79% (98/124 patients) in patients receiving ciprofloxacin; this was significantly lower in patients not treated with ciprofloxacin (40%, 6/15 patients) (181).

Respiratory tract infections in CF. In a retrospective study involving 110 CF patients (infected with different microorganisms), patients treated with antibiotics that were found to be active against biofilm-grown bacteria *in vitro* showed a significant reduction in the sputum bacterial density, a significant reduction in length of hospital stay and a non-significant decrease in treatment failure (182). However, the only two randomized clinical studies addressing the added value of using antibiotics with activity against biofilms yielded no evidence for choosing antibiotics based on biofilm AST for the treatment of *P. aeruginosa* respiratory tract infections in people with CF (183). In the first study (184), 39 patients were randomized to biofilm or conventional treatment groups, in which antibiotics were selected based on biofilm susceptibility testing with the Calgary biofilm device and broth susceptibility testing, respectively. However, no microbiological or clinical differences were observed between both groups. In the second study (185), the effect of 14 days of intravenous antibiotic treatment for pulmonary exacerbations due to *P. aeruginosa* was compared between patients receiving treatment based on conventional or biofilm antimicrobial susceptibility results. Also in this study no differences in microbiological (sputum density at day 14 of the treatment and at the 1 month follow-up visit) or lung function parameters could be observed between both groups.

Potential explanations for the lack of association between biofilm susceptibility and clinical outcome. While large randomized clinical trials about the use of biofilm-active antibiotics in prosthetic joint infections are lacking, the data summarized above seem to indicate an added value of using biofilm-active antibiotics in this context, suggesting that predicting which antibiotics would have activity against biofilms (especially in the context of 'difficult-to-treat' infections and/or infections caused by less-frequently encountered pathogens) could lead to an improved outcome (although the apparently conflicting data about biofilm-activity of rifampicin remains to be settled). The situation is however different in the context of biofilm-related respiratory tract infections in CF, where two randomized clinical trials could not find an added value of biofilm-based susceptibility testing, despite promising data in a retrospective study (182). While it cannot be ruled out that the very different etiology of prosthetic joint infections and respiratory tract infections in CF is behind this apparent discrepancy, it should be noted that in the two clinical trials in CF patients, biofilm susceptibility was determined using the Calgary biofilm device and cation-adjusted Mueller-Hinton broth as growth medium (105, 184, 185). In this model biofilms will develop as surface-attached communities in a growth medium that is physico-chemically very different from CF sputum. However, we know that the microenvironment plays an important role in various aspects of biofilm biology (including metabolism) and likely has a profound impact on antimicrobial susceptibility (13, 26, 160, 186, 187). It should thus maybe not come as a surprise that biofilm susceptibility testing in an *in vitro* model that is poorly representative of the *in vivo* situation, yields susceptibility data that are poorly representative of the activity of the antibiotic against *in vivo* biofilms (114, 188); indeed, such tests may not be a better predictor of *in vivo* anti-biofilm activity than planktonic susceptibility tests.

HOW CAN WE IMPROVE BIOFILM SUSCEPTIBILITY TESTING AND MAKE IT MORE RELEVANT FOR CLINICAL PRACTICE?

The Importance of Standardization and Use of Appropriate Parameters

In order for biofilm AST to find its way to clinical practice, substantial standardization will be required in order to obtain methods that are reproducible and repeatable, and yield susceptibility data that are in categorical agreement, regardless of the place where they were obtained (114). Standardization and reproducibility in biofilm research has been receiving increasing attention, especially (but not exclusively) in the context of developing products or devices with anti-biofilm activity (114-116, 120, 137, 188-192). The recent launch of an International Biofilm Standards Task

Group (https://www.biofilms.ac.uk/international-standards-task-group/) is in line with this increased attention for standards. The challenge of developing standardized biofilm susceptibility tests should not be underestimated. Biofilm-based assays are inherently more complex than assays based on planktonic cells, and even results from these (technically less-demanding) conventional susceptibility tests are influenced by minor deviations from the published reference methods, again highlighting the need for standardization and adequate quality control (34, 193-196). While many factors influence the outcome of a biofilm experiment, results from several studies suggest that how the biofilm is grown and how the inoculum is prepared are crucial (115, 197-199), and that reproducibility between laboratories improves when a common (standardized) protocol is used (115).

However, prior to standardization, there needs to be a consensus on which pharmacodynamic parameter(s) (Table 1; Fig. 2) is (are) the most important. It could be argued that in line with planktonic susceptibility testing, we first and foremost want to know which antibiotic will affect the development of a biofilm, but whether this pertains to the development starting from a planktonic culture (i.e. *prevention* of biofilm formation, parameter: BPC) or from a young biofilm (i.e. *inhibition* of progression of biofilm formation, parameter: MBIC) is open for discussion. It is currently unclear whether biofilm-associated infections are initiated by the introduction of single cells, aggregates or both (1), but regardless of this, it seems in most cases unlikely that antibiotic therapy would be started so quickly after the introduction of the organisms that no aggregates would be present at the start of the treatment (even if the infection was initiated by single cells), which would argue for the use of MBIC as parameter. An exception to this would be antibiotic therapy started prior, during, or immediately after surgery in which case the presence of single cells or very small aggregates is more likely. In many cases, antibiotic therapy will only be started after the patient starts showing symptoms, and this means that in most cases biofilm aggregates will already have formed. This implies that it is also important to know which concentrations of an antibiotic will lead to partial reduction (i.e. a reduction in biofilm, but not complete eradication) or full eradication. For the latter the MBEC is an appropriate parameter, while the MCK-x (i.e. the concentration required to achieve x-log reduction) can be used for the former. Finally, biofilm tolerance factors (BTF-I, BTF-E, BTF-x; Table 1) could be used to quantify biofilm-related reduced susceptibility in comparison to susceptibility of planktonic cells (110).

The proposed definitions in Table 1 are independent of the analysis method used and are (at least in theory) equally valid for different biofilm quantification approaches. However, in the context of biofilm AST, approaches that directly (e.g. plate counts) or indirectly (e.g. resazurin-based viability staining, ATP measurements) quantify the number of living and/or culturable cells will likely be preferred over methods that only provide crude measurements of biofilm biomass (e.g. biofilm biomass staining with crystal violet).

Setting of Biofilm Breakpoints

Breakpoints are used to distinguish between 'susceptible' organisms ('susceptible' implying that the use of a particular antibiotic for this organism is associated with a high likelihood of therapeutic success) and 'resistant' organisms ('resistance' implying that the use of this particular antibiotic for an infection caused by this organism is typically associated with clinical failure) (33, 200). These breakpoints are set by organizations like EUCAST and CLSI and take into account a wide range of parameters, including data from large-scale clinical studies, wild-type MIC distributions, and PK/PD aspects (33, 35, 36, 201-203). As none of these data are currently available for biofilm infections, setting biofilm breakpoints will be far from trivial and as already mentioned above, there is no evidence for an added value of using planktonic breakpoints to categorize biofilms as 'susceptible' or 'resistant'. Recently a potential solution was proposed for the lack of biofilm breakpoints, i.e. determining epidemiological cut-off (ECOFF) values (MBIC-ECOFF and MBEC-ECOFF) to distinguish between strains belonging to the wild-type population and strains belonging to the population possessing acquired mechanisms responsible for reduced antimicrobial susceptibility of biofilms (204). This approach is in line with the EUCAST recommendations for setting breakpoints for the topical use of antimicrobial agents and the use of inhaled antibiotics (205). Of course, establishing such ECOFFs would only be the first step, and biofilm breakpoints should ultimately be based on data from large clinical studies.

Increasing the Biological Relevance of *In Vitro* Tests

We know that the nutritional environment can influence results of conventional AST and several attempts have been made to increase the biological relevance of *in vitro* AST by re-creating the *in vivo* conditions *in vitro* (104, 158, 206-212). However, in the absence of a thorough validation it is unclear whether these modified test conditions really are more *in vivo*-like and it is often also unclear whether microorganisms grown in these systems reflect the *in vivo* biofilm phenotype.

Many different artificial or synthetic sputum media, mimicking the composition of CF sputum have been developed (213-216) and it is also in this context that the '*in vivo*-likeness' of at least some media has been evaluated to the greatest extent, both in terms of gene expression (45, 47) and in terms of morphological similarity between *in vitro* and *in vivo P. aeruginosa* aggregates (217). Likewise, substantial efforts have been made to develop growth media that better represent the *in vivo* microenvironment of a prosthetic joint infection, mainly based on the addition of human or animal synovial fluid, or the development of synthetic synovial fluid (218-226) (Fig. 3). Most of the work done in these media so far has focused on studying the formation of biofilm aggregates in various staphylococci, but some of the media developed have been used to asses biofilm antimicrobial susceptibility as well (219, 220, 222). Finally, a range of relevant models for the study of infected wounds have been developed that allow to study antimicrobial treatments of these biofilm-related infections under *in vivo* or *in vivo*-like conditions (227-234).

The Need for Clinical Trials to Validate the Use of Biofilm-based Susceptibility Testing in Clinical Practice

Even if we manage to develop standardized and physiologically relevant *in vivo*-like biofilm models that can be incorporated in the workflow of a clinical microbiology lab, their success will ultimately depend on whether using them improves the clinical outcome of a treatment.

The added value of biofilm-based AST for treating a specific biofilm-related infection could be determined in a clinical trial in which patients are randomized to a 'conventional treatment group' (in which antibiotic treatment is selected based on conventional susceptibility testing) and a 'biofilm treatment group' (in which antibiotic treatment is selected based on biofilm-based susceptibility testing), much like was done for CF (184, 185). A protocol of a proposed prospective randomized clinical trial for selection of antibiotics in periprosthetic joint infections guided by MBEC and MIC determinations was recently published (235). This trial aims to include patients with first-time prosthetic joint (hip or knee) infections (monomicrobial infections with *Staphylococcus* spp.) and its primary outcome measurement is the proportion of changes in antimicrobial regimen from first-line treatment. The trial aims to recruit 64 patients that will be randomized to a standard of care arm (choice of antibiotic guided by MIC) or a comparative arm (selection of antibiotics based on MIC and MBEC) (235).

However, setting up such a randomized controlled trial, with a sufficiently-high number of patients in each group and clearly-defined endpoints, will be challenging. Obtaining ethical approval might also be difficult, either because it is accepted by many that a particular antibiotic is superior to others, e.g. in the case of rifampicin for treating prosthetic joint infections (178), or because of the disappointing outcomes in earlier trials, e.g. in CF (184, 185). Finally, for many biofilm-related infection (including wound infections and prosthetic joint infections), administration of antibiotics is only a part of the treatment and variations in other interventions (e.g. surgical debridement, one-or two-stage revision surgery) will complicate recruitment, randomization and interpretation of the outcome (236). Considering these difficulties, a more feasible alternative approach could be envisaged in which the antibiofilm activity of antibiotics is determined in one or more optimized models in order to devise treatment regimens with potential *in vivo* activity against biofilms. In a second step, the clinical outcome of these biofilm-active regimens can then be compared to the outcome observed with conventional therapy (i.e. therapy with antibiotics selected based on conventional AST).

The results obtained such studies will allow to build a knowledge base for further research that could ultimately pave the way for a broader introduction of these approaches in the clinical microbiology laboratory.

Practical Aspects

The success of biofilm-based AST in the clinical laboratory will also depend on the development and implementation of affordable, reproducible and high-throughput tools that yield results that are easy to interpret, as it seems very unlikely that methods based on complex low-throughput biofilm model systems, using expensive advanced approaches for readouts, and/or requiring extensive hands-on time, will find their way to clinical practice. However, the highly successful introduction of MALDI-TOF mass spectrometry for rapid and accurate identification of microorganisms in the clinical microbiology laboratory (237-240) shows that the development and implementation of advanced methodology is possible. While it is at this point difficult to predict what exactly will be needed, it will likely involve the development of validated and standardized pre-made relevant media to grow biofilms and the development and implementation of automated and high-throughput methods for reading biofilm susceptibility. Regardless of what form biofilm-based AST ultimately will take, the successful implementation will require collaboration between basic researchers, clinical microbiology laboratories and (potentially new) companies involved in developing and marketing diagnostic tools.

CONCLUDING REMARKS

The call for bringing biofilm AST to the clinic is not new. Already in 2006, Sandoe *et al* wrote that '*Data from large numbers of clinical episodes would be required to define the relationship between MBIC and clinical outcome before any advantages over MIC could be assessed. We hope that this work will stimulate the investigation of susceptibility tests that have more relevance to biofilm infections than current methods.*' (241). Our profound knowledge about biofilm formation (1), our insights into mechanisms responsible for reduced susceptibility in biofilms (25, 86) and the realization that the infectious microenvironment plays a crucial role in antimicrobial susceptibility

(26), will be essential to develop and validate relevant biofilm-based AST methods that can be used in clinical microbiology laboratories. The crucial next step will be the evaluation of these methods in well-designed clinical trials, with as ultimate goal to improve antibiotic treatment of patients suffering from biofilm-related infections.

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TABLE 1. Proposed key pharmacodynamic parameters that could be used as measures for biofilm susceptibility and their definition. Information in this table is partially based on (but not necessarily equal to) definitions proposed previously (107, 109-111, 113).

	Parameter	Abbreviation	Proposed definition/comment[a]
Prevention	Biofilm prevention concentration	BPC	Lowest concentration of an antibiotic required to fully prevent formation of a biofilm (including biofilm aggregates) starting from planktonic cells
Inhibition	Minimal biofilm inhibitory concentration	MBIC	Lowest concentration of an antibiotic required to fully prevent the further development of a biofilm
Eradication	Minimal biofilm eradication concentration	MBEC	Lowest concentration of an antibiotic required to fully eradicate an established biofilm (i.e. resulting in a read-out below the detection limit)
Killing	Minimum antibiotic concentration for biofilm killing to achieve x-log reduction[b]	MCBK-x	Lowest concentration of an antibiotic required to achieve x-log reduction in an established biofilm[c]
Relative parameters	Biofilm tolerance[d] factor-prevention	BTF-P	The ratio of the BPC and the MIC
	Biofilm tolerance factor-inhibition	BTF-I	The ratio of the MBIC and the MIC
	Biofilm tolerance factor-eradication	BTF-E	The ratio of the MBEC and the MIC
	Biofilm tolerance factor-x	BTF-x	The ratio of the MCBK-x and the MIC

[a] The definitions are proposed in general terms, i.e. independent of a specific quantification method.

[b] The word 'biofilm' was added to the definition previously proposed (110) to avoid any confusion.

[c] The MCBK resulting in complete eradication is equal to the MBEC.

[d] For an in-depth discussion and definition of tolerance see references (25, 155, 242-245).

FIGURE 1. A. Association between biofilm-forming capacity and resistance to specific antibiotics in a collection of 299 *Staphylococcus* spp. strains; *: p< 0.05. Only for rifampicin a significant association between increased biofilm formation (assessed by crystal violet staining) and resistance was observed. Based on data reported in (141). Abbreviations: FOX, cefoxitin; ERY, erythromycin; CLI, clindamycin; NOR, norfloxacin; GEN, gentamicin; SXT, sulfamethoxazole/trimethoprim; TIG, tigecycline; LZD, linezolid; FUS, fusidic acid; RIF, rifampicin; VAN, vancomycin. **B.** Association between planktonic (MIC) and biofilm (BPC) susceptibility towards three antibiotics for nine *P. aeruginosa* isolates. The yellow line indicates the situation in which both parameters would be identical. While the BPC is always higher than the MIC, exact BPC values cannot be predicted based on MIC. Based on data reported in (160). Abbreviations: TOB, tobramycin; CIP, ciprofloxacin; COL, colistin.

FIGURE 2. Illustration of key pharmacodynamic parameters that could be used as measures for biofilm susceptibility. MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; BPC, biofilm prevention concentration; MBIC, minimal biofilm inhibitory concentration; MBEC, minimal biofilm eradication concentration.

FIGURE 3. A. *P. aeruginosa* biofilm aggregate grown in SCFM2 medium. **B.** *S. aureus* biofilm aggregate grown in synthetic synovial fluid medium. **C.** Biofilm prevention concentration of three antibiotics against nine *P. aeruginosa* biofilms (A-I) determined in SCFM2 (based on data reported in (160)).

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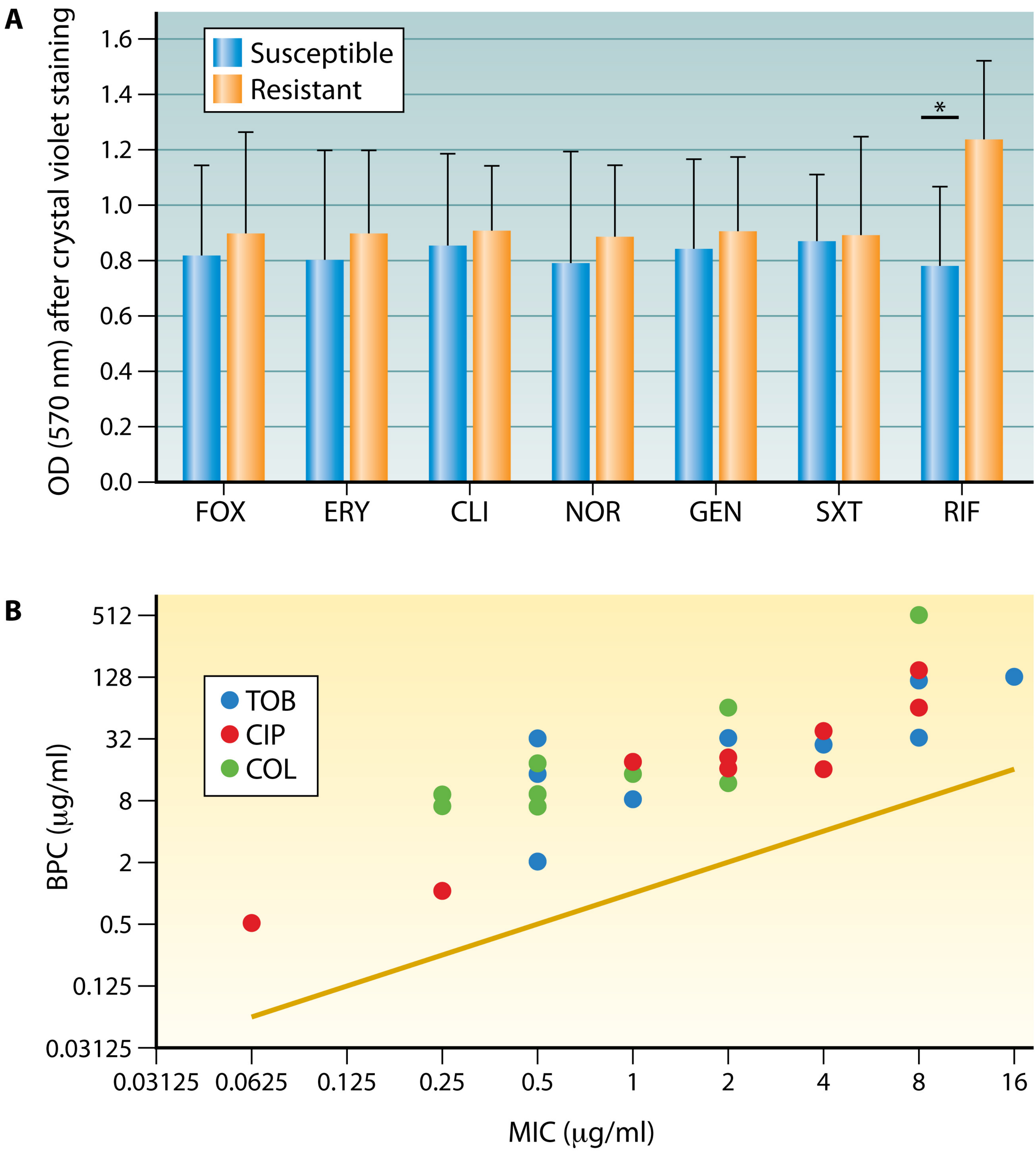
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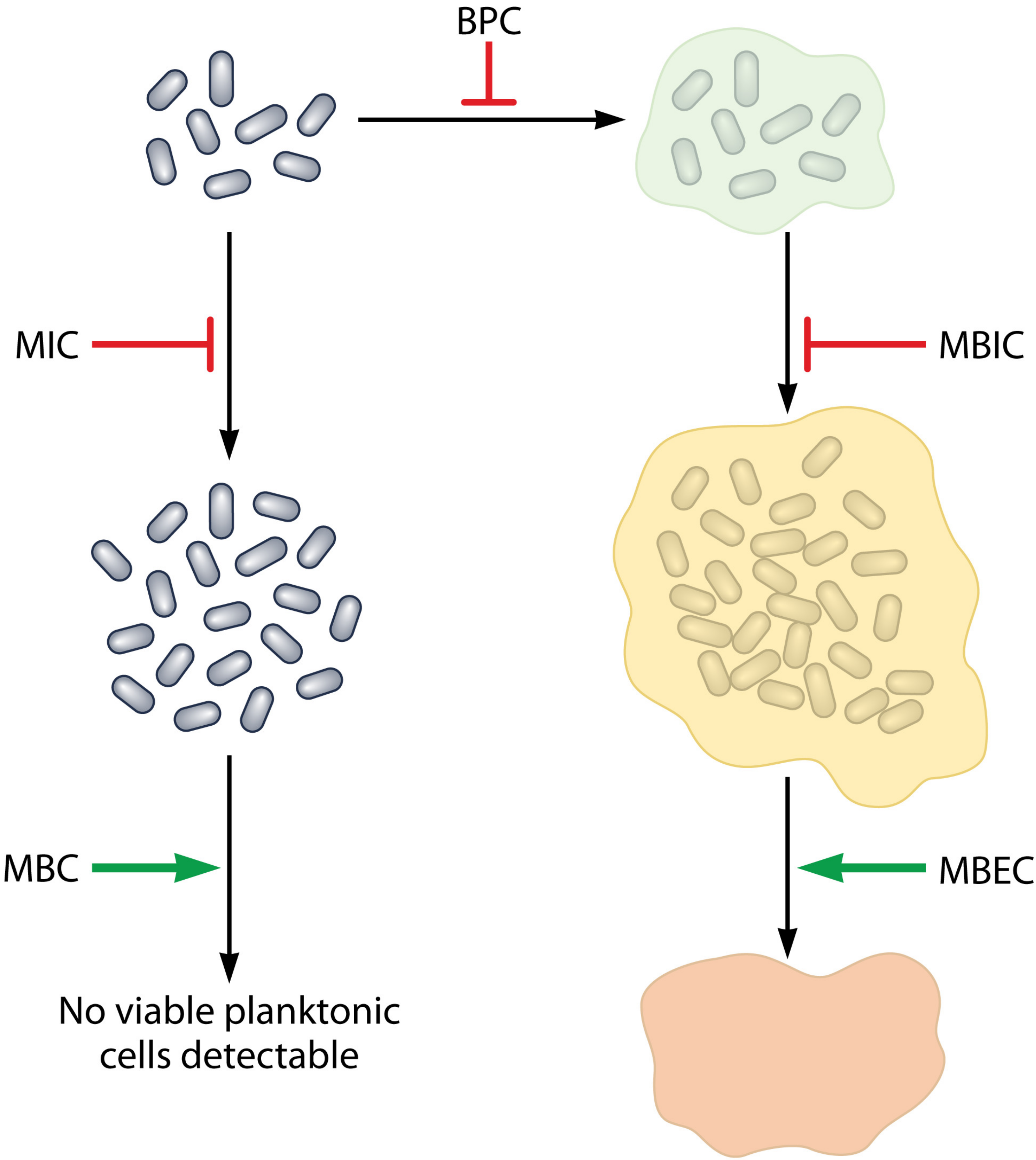
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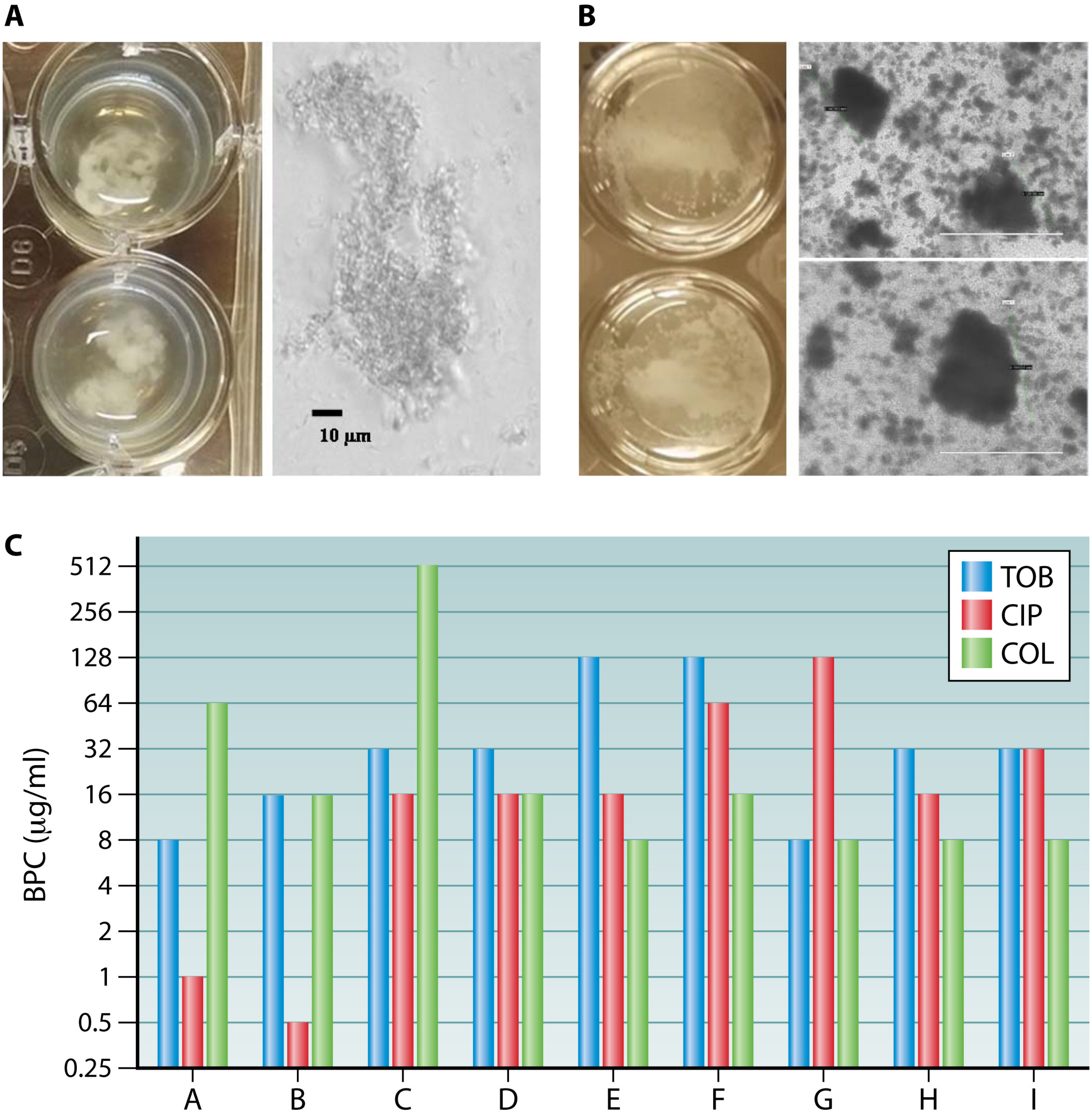

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