Video Article

Use of Artificial Sputum Medium to Test Antibiotic Efficacy Against *Pseudomonas aeruginosa* in Conditions More Relevant to the Cystic Fibrosis Lung

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Abstract

There is growing concern about the relevance of *in vitro* antimicrobial susceptibility tests when applied to isolates of *P. aeruginosa* from cystic fibrosis (CF) patients. Existing methods rely on single or a few isolates grown aerobically and planktonically. Predetermined cut-offs are used to define whether the bacteria are sensitive or resistant to any given antibiotic[1]. However, during chronic lung infections in CF, *P. aeruginosa* populations exist in biofilms and there is evidence that the environment is largely microaerophilic[2]. The stark difference in conditions between bacteria in the lung and those during diagnostic testing has called into question the reliability and even relevance of these tests[3].

Artificial sputum medium (ASM) is a culture medium containing the components of CF patient sputum, including amino acids, mucin and free DNA. *P. aeruginosa* growth in ASM mimics growth during CF infections, with the formation of self-aggregating biofilm structures and population divergence[4,5,6]. The aim of this study was to develop a microtitre-plate assay to study antimicrobial susceptibility of *P. aeruginosa* based on growth in ASM, which is applicable to both microaerophilic and aerobic conditions.

An ASM assay was developed in a microtitre plate format. *P. aeruginosa* biofilms were allowed to develop for 3 days prior to incubation with antimicrobial agents at different concentrations for 24 hours. After biofilm disruption, cell viability was measured by staining with resazurin. This assay was used to ascertain the sessile cell minimum inhibitory concentration (SMIC) of tobramycin for 15 different *P. aeruginosa* isolates under aerobic and microaerophilic conditions and SMIC values were compared to those obtained with standard broth growth. Whilst there was some evidence for increased MIC values for isolates grown in ASM when compared to their planktonic counterparts, the biggest differences were found with bacteria tested in microaerophilic conditions, which showed a much increased resistance up to a >128 fold, towards tobramycin in the ASM system when compared to assays carried out in aerobic conditions.

The lack of association between current susceptibility testing methods and clinical outcome has questioned the validity of current methods[3]. Several in *vitro* models have been used previously to study *P. aeruginosa* biofilms[7, 8]. However, these methods rely on surface attached biofilms, whereas the ASM biofilms resemble those observed in the CF lung[9] . In addition, reduced oxygen concentration in the mucus has been shown to alter the behavior of *P. aeruginosa*[2] and affect antibiotic susceptibility[10]. Therefore using ASM under microaerophilic conditions may provide a more realistic environment in which to study antimicrobial susceptibility.

Video Link

The video component of this article can be found at https://www.jove.com/video/3857/

Protocol

1. Preparation of Artificial Sputum Medium (ASM)

1. Add 4 g DNA from fish sperm to 250 ml sterile water very slowly over a period of several hours. The DNA takes several hours to completely dissolve and can be stirred overnight at room temperature.
2. Add 5 g mucin from porcine stomach (type II) slowly to 250 ml sterile water until the mucin has dissolved completely. The solution can be stirred overnight at 4 °C.
3. Dissolve 0.25 g of each essential and non-essential L-amino acid, with the exception of L-tyrosine and L-cysteine, in 100 ml sterile water. Dissolve 0.25 g of L-cysteine in 25 ml of 0.5 M potassium hydroxide (M_r 56.11 g/mol) and 0.25 g of L-tyrosine in 25 ml sterile water.
4. Dissolve 5.9 mg diethylenetriaminepentaacetic acid (DTPA), 5 g NaCl and 2.2 g of KCl in 100 ml of sterile water.
5. Combine the DNA, Mucin, L-amino acids, DTPA, NaCl and KCl in a 1 litre bottle.

6. Add 5 ml of egg yolk emulsion and fill to approximately 850 ml with sterile water.
7. Adjust pH to 6.9 with 1 M Tris (pH 8.5; M_r 121.14) and bring the volume to 1 litre with sterile water.
8. Sterilize the ASM by filtration using a Vacuubrand ME 2 diaphragm vacuum pump and Millipore Steritop filter units with a pore and neck size of 0.22 µm and 45 mm, respectively. Each Steritop filter unit can be re-used immediately up to three times; however, the filters need to be rinsed twice with sterile water before re-use. The filtration process is slow and can be performed over 2 days. Other versions of ASM have been developed that use the addition of antibiotics instead of filtration[11] however, due to possible drug interactions, we do not recommend the method for this particular application.
9. Unfiltered and filtered ASM should be stored at 4 °C in the dark. Using fresh ASM is recommended however, it can be kept under these conditions for a maximum of one month.

2. Determination of the Planktonic Sessile Cell Minimum Metabolic Inhibitory Concentration (PSMIC)

1. To determine the minimum metabolic inhibitory concentration (PSMIC) values for 15 planktonically grown *P. aeruginosa* isolates, the microdilution method should be performed, as described in the guidelines of the British Society for Antimicrobial Chemotherapy BSAC[12]. The antibiotic of choice, in this case tobramycin sulphate, is serially diluted in Luria-Bertani (LB) medium in a 96-well microtitre plate to provide an appropriate range of antibiotic concentrations.
2. Dilute overnight cultures of *P. aeruginosa* in LB to an OD_{600} of 0.05 (±0.01) and add 100 µl volumes to the wells of the 96-well microtitre plate containing 100 µl of the serially diluted antibiotic. In this case, the final concentrations of tobramycin sulphate ranged between 512 - 0.5 µg/ml. Eight replicates of each antibiotic concentration should be performed.
3. Negative control wells for each isolate should be set up, in which no antibiotic is added. Also, eight wells should contain only LB for use as a blank during downstream analysis (Section 2.6).
4. Incubate the 96-well microtitre plates for 1 - 2 days at 37 °C without shaking under aerobic or microaerophilic (5 % O_2, 10 % CO_2, and 85 % N_2) conditions. Microaerophilic conditions are obtained using CampyGen gas generation packs in large anaerobic jars.
5. Following incubation, bacterial growth is determined by measuring the absorbance of the bacterial culture in each well at a wavelength of 600 nm using a Fluostar Omega microplate reader and the MARS Data Analysis Software.
6. Absorption from antibiotic-treated planktonic cultures ($A_{\text{antibiotic treated planktonic cells}}$) and absorption from the negative controls ($A_{\text{negative control}}$) should be corrected by subtraction of the background absorbance obtained from the wells containing LB only (A_{blank}). The percentage inhibition of viability is subsequently calculated as (mean $A_{\text{antibiotic treated planktonic cells}}$/mean $A_{\text{negative control}}$)x 100 %. The $PSMIC_{90}$ is defined as the antibiotic concentration causing 90 % inhibition of planktonic bacterial growth.
7. To determine bacterial viability after treatment with the antibiotic of choice, 10 µl of 0.02 % (v/v) resazurin (diluted in distilled water) is added to each well and the plates are incubated under aerobic conditions for 1 - 2 h at 37 °C, while shaking at 150 rpm. Viable cells will reduce the blue resazurin dye to the pink fluorescent resorufin form.
8. Following incubation with resazurin, monitor the fluorescence of each well using an excitation wavelength of 540 nm and an emission wavelength of 590 nm in a Fluostar Omega microplate reader. The data should be analysed as described below.

3. Determination of Biofilm Sessile Cell Minimum Inhibitory Concentration (BSMIC)

1. Overnight cultures of *P. aeruginosa* (in this case, 15 isolates of *P. aeruginosa* are used) should be diluted in LB to an OD_{600} of 0.05 (±0.01), then further diluted 1:100 in fresh ASM (total volume 1.8 ml).
2. The diluted cultures (1.8 ml) should be added to each well of a 24-well tissue culture treated plate. Three wells should contain ASM only for use as a blank during downstream analysis (Section 3.9).
3. Secure the 24-well plates with laboratory parafilm and incubate for 3 days under aerobic or microaerophilic conditions at 37 °C, while shaking at 75 rpm. Microaerophilic conditions should be obtained using CampyGen gas generation packs in large anaerobic jars.
4. Dilute the antibiotic of choice, in this case tobramycin sulphate, to provide an appropriate concentration range in fresh ASM. In this instance, final concentrations ranged between 512 - 1 µg/ml. Add each concentration of the antibiotic, in volumes of 200 µl, to the appropriate wells of the 24-well plates. Four replicates of each antibiotic concentration should be performed. Biofilms not exposed to the antibiotic of choice were used as a negative control.
5. Secure the 24-well plates with laboratory parafilm and incubate under aerobic or microaerophilic conditions for a further 24 h at 37 °C, while shaking at 75 rpm.
6. After incubation in the presence of the antibiotic of choice, disrupt the bacterial biofilms using 100 µl of 100 mg/ml cellulase (diluted in 0.05 M citrate buffer [9.6 g/l Citrate.H_20 in water and pH to 4.6 with NaOH]) and incubate the 24-well plates under aerobic conditions at 37 °C, while shaking at 150 rpm for 1 h. If required, biofilms could be further disrupted by manual pipetting at this stage.
7. To determine the metabolic activities of the bacterial cells released from the disrupted biofilms, 100 µl of 0.02 % (v/v) resazurin (diluted in distilled water) should be added to each well of the 24-well plates and incubated for 1 - 2 h at 37 °C, while shaking at 150 rpm.
8. Following incubation with resazurin, measure the fluorescence of each well using an excitation wavelength of 540 nm and an emission wavelength of 590 nm in a Fluostar Omega microplate reader and the MARS Data Analysis Software.
9. Fluorescence from the antibiotic-treated biofilms ($F_{\text{antibiotic-treated biofilms}}$) and fluorescence from the negative controls ($F_{\text{negative control}}$) should be corrected by subtraction of the background fluorescence obtained from the wells containing ASM only (F_{blank}). The percentage inhibition of viability is subsequently calculated as (mean of $F_{\text{antibiotic treated biofilms}}$/mean $F_{\text{negative control}}$) x 100 %. The $BSMIC_{90}$ is defined as the antibiotic concentration causing 90 % inhibition of metabolic activity.

4. Representative Results

ASM biofilm formation is possible in small (2 ml) volumes and the biofilms are fully formed within 3 days (**Figure 1A**). This can be demonstrated by rigorously pipetting the biofilm, which should be difficult to disrupt. The microcolonies are comparable to those grown in larger volumes[4] (**Figure 1B**). **Figure 2** shows major differences between cells grown planktonically and in a biofilm as detected by electron microscopic image

analysis. Biofilm cultures clearly show considerable levels of extracellular matrix surrounding the cells and individual structures within the biofilm are difficult to identify.

Several studies suggest that the biofilm lifestyle can affect antimicrobial susceptibility[13, 14]. Our small scale ASM assay can be used to determine the BSMIC of multiple antibiotics for multiple isolates at the same time. The workflow of the assay is shown in **Figure 3**. The effect of antibiotics on bacterial cell viability can be measured using the resazurin assay. Antibiotics, in this case tobramycin, can be added to the established biofilm and incubated for 24 h. After this the biofilm is disrupted and resazurin is added.

Metabolically active cells can reduce the resazurin dye resulting in a colour change from blue (resazurin) to pink (resorufin) [15]. **Figure 4A** shows an example assay in which *P. aeruginosa* was incubated with different concentrations of tobramycin before biofilm disruption and addition of resazurin in a microtitre plate. The blue non-fluorescent colour indicates non-viable cells, whereas viable cells reduce the dye to the pink fluorescent form, resorufin. The SMIC can then be calculated by converting fluorescence into percentage remaining bacterial viability. **Figure 4B** shows the change in % viability with increasing tobramycin concentration. 10% viability was chosen as a cut-off in order to calculate the $SMIC_{90}$.

Under aerobic conditions, the tobramycin $SMIC_{90}$ values are higher for cells grown as a biofilm than those of planktonic cultures. Table 1 shows the variation in $PSMIC_{90}$ and $BSMIC_{90}$ for all isolates tested. Table 2 shows that under aerobic conditions, a dramatic increase in resistance to tobramycin (2 to >32 fold increase in SMIC) was observed for most isolates when grown in ASM (biofilm mode) compared to LB (planktonic mode). In addition, biofilms grown under microaerophilic conditions exhibited an increased SMIC of between 2 and >128-fold when compared to biofilms grown under aerobic conditions.

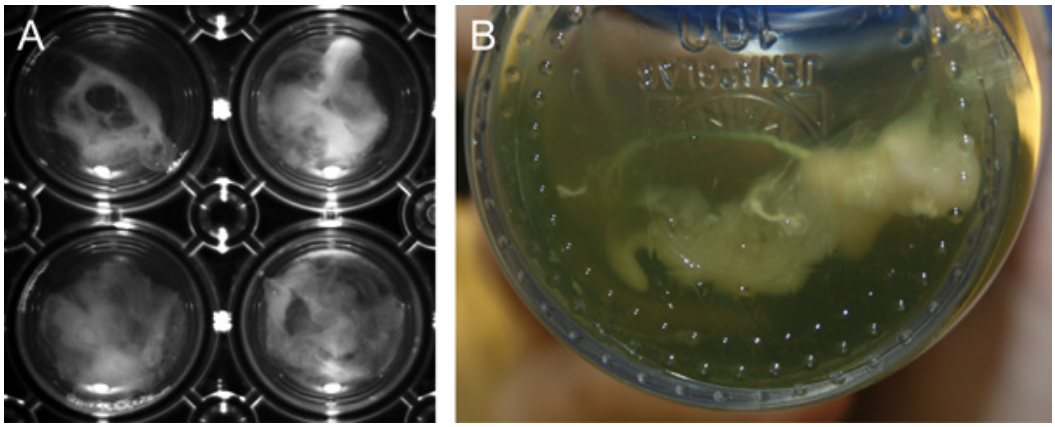

Figure 1. Biofilm formation of P. aeruginosa in ASM *P. aeruginosa* strain PAO1 forms macroscopically visible clumps (microcolonies) when grown in ASM. **A**, Biofilm formation in 30 ml ASM cultures (large-scale) after 7 days growth in screw cap glass Duran flasks . **B**, Biofilm formation in 2 ml ASM cultures (small-scale) after 3 days growth in 24-well polystyrene plates.

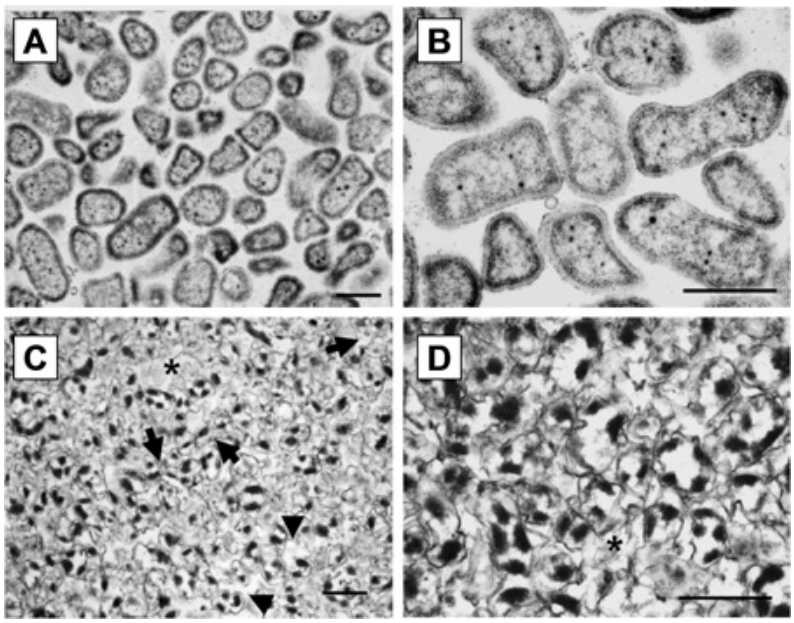

Figure 2. TEM micrographs of ASM biofilms A/C TEM micrograph (x;27,000) of PAO1 grown planktonically and in ASM, respectively, **B/D** TEM micrograph (x57,000) of PAO1grown planktonic and in ASM, respectively. Planktonically grown bacteria were cultivated overnight in LB broth. Biofilms were cultivated for 7 days in 30 ml ASM cultures. Black arrows refer to cells within the biofilm and stars refer to extracellular spaces. Scale bars = 1 µm.

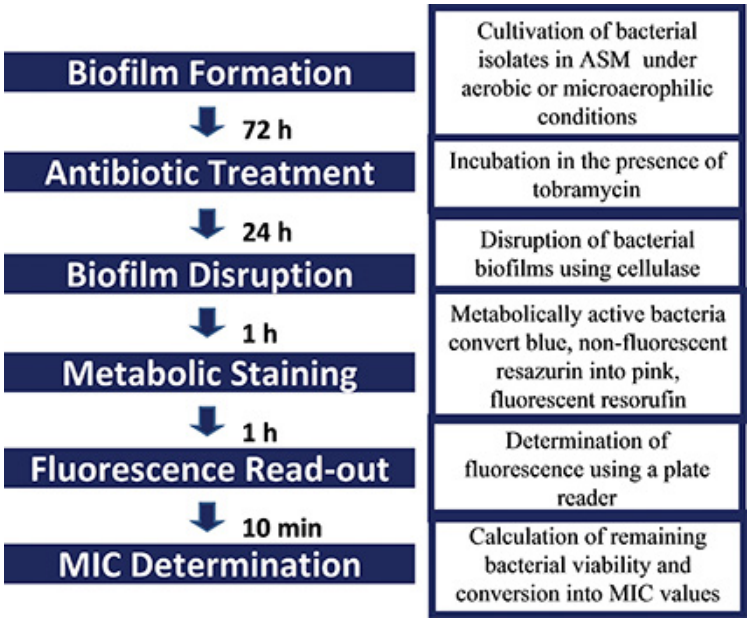

Figure 3. Workflow of the ASM biofilm antimicrobial susceptibility assay.

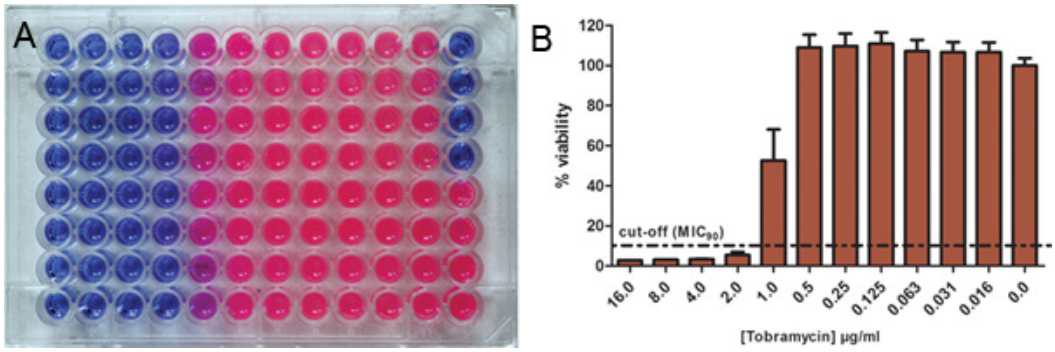

Figure 4. Use of resazurin for the determination of antibiotic susceptibilities Bacterial cells were incubated with different concentrations of the antibiotic and the remaining metabolic activity was determined using resazurin. **A**, The blue non-fluorescent oxidized form of resazurin indicates non-viable cells and is reduced by metabolic active cells to pink fluorescent resorufin. **B**, Fluorescence intensity is converted into percentage of remaining bacterial viability. 10% viability was chosen as cut-off in order to calculate the MSMIC90. Click here to view larger figure.

Strains	PSMIC $_{90}$ (µg/ml) [1]		BSMIC $_{90}$ (µg/ml) [1]	
	Aerobic	Microaerophilic[2]	Aerobic	Microaerophilic [2]
PAO1	4	4	8	>512
Liverpool Epidemic Strain (LES) isolates				
LESB58[21]	8	64	64	128
LES400[22]	32	128	8	256
LESB25	16	32	256	512
LESB55	16	64	64	>512
LESB64	16	64	>512	>512
LES431[22]	4	8	32	>512
LESB49	16	64	64	256
LES109	32	128	32	>512
Non-LES isolates				
49461	16	32	16	>512
59032	0.5	2	4	>512
59073	>512	>512	>512	>512
59076	16	32	32	>512
27	8	16	4	>512
45	16	32	4	>512

Table 1. Susceptibility of P. aeruginosa to tobramycin.

[1]For determination of PSMICs and BSMICs tobramycin was used in 2-fold serial dilutions ranging from 512 - 0.5 µg/ml (n = 8 for each concentration) and 512 - 1 µg/ml (n = 4 for each concentration), respectively;; PSMICs were determined using the standard microdilution method[1].

[2]Microaerophilic conditions were 5 % O_2, 10 % CO_2, and 85 % N_2.

Strain	$PSMIC_{90}/BSMIC_{90}$ fold change[1]			
	$PSMIC_{aerobic} \rightarrow PSMIC_{microaerophilic}$	$BSMIC_{aerobic} \rightarrow BSMIC_{microaerophilic}$	$PSMIC_{aerobic} \rightarrow BSMIC_{aerobic}$	$PSMIC_{microaerophilic} \rightarrow BSMIC_{microaerophilic}$
PAO1	0	**>64**	2	**128**
LES isolates				
LESB58	8	2	8	2
LES400	4	**32**	0.25	2
LESB25	2	2	**16**	**16**
LESB55	4	>8	4	>8
LESB64	4	ND	**>32**	>8
LES431	2	**>16**	8	**>64**
LESB49	4	4	4	4
LES109	4	**16**	0	>4
Non-LES isolates				
49461	2	**>32**	0	**>16**
59032	4	**>128**	8	**>256**

59073	ND	ND	ND	ND
59076	2	**>16**	2	**>16**
27	2	**>128**	0.5	**>32**
45	2	**>128**	0.25	**>16**

Table 2. Fold change of PSMICs and BSMICs to tobramycin.

[1]ND, not determined; values in bold indicate SMIC fold changes >10.

Discussion

In this study we used a novel *in vitro* model based on ASM to replicate *P. aeruginosa* biofilm conditions within the CF lung[4]. The model was modified successfully for small-scale, high-throughput testing of antimicrobial agents.

The critical steps of this assay are:

1. Consistent preparation of the ASM media and maintaining sterility. We have devoted long hours to optimising the way in which each component is added to achieve reproducible results every time. Filtration of the ASM is slow but is preferable to autoclaving, which may damage the mucin component. We do not advise the addition of antibiotics, as suggested by others [11] because this may impose considerable selective pressures, drive mutations, induce prophage lysis [16] and significantly alter the expression of multiple bacterial genes.
2. The assay should be optimised according to the volume of ASM used. Shaking speeds are increased for smaller volumes and a shorter biofilm life-cycle is observed.

An obvious application of the small-scale ASM biofilm model is the more realistic determination of biofilm antimicrobial susceptibilities ($BSMIC_{90}$). Anaerobic and microaerophilic niches are present in the CF lung and there is evidence that oxygen is limited deep within mature biofilms[2, 17]. Here we demonstrate that 10/14 clinical *P. aeruginosa isolates* from CF patient sputa exhibit a considerable (4 - ≥ 128 fold) decrease in sensitivity to tobramycin under microaerophilic conditions in ASM. The results of this study suggest that antibiotics, such as tobramycin, might be less effective against *P. aeruginosa* infections in the CF lung than indicated by conventional susceptibility testing methods. These results reflect previous studies on the antimicrobial susceptibility of biofilms [10]. Small-scale ASM assays thus provide a simple high throughput platform for generating meaningful antibiotic susceptibility data to better inform therapeutic decisions. The assay is limited in the same way as conventional antibiotic susceptibility testing in that single colonies are picked for screening that may not be representative of the whole population. However, we believe that an approach (i) using non-surface attached biofilm growth and (ii) applicable to microaerophilic conditions, represents a clear alternative and a potential improvement to existing methods. We conclude that this assay is an appropriate model to study *P. aeruginosa* biofilm populations. Further testing in clinical settings would ascertain whether antibiotic susceptibilities based on biofilm-grown *P. aeruginosa* could lead to different antibiotic choices with potentially improved microbiological and clinical outcomes. Similar investigations using classic biofilm models have shown that BSMIC values lead to different recommendations for antibiotic treatment[5,17].

In addition to testing for the effectiveness of anti-infective agents, the ASM system represents a cheap, simple and reproducible alternative to animal models for studies such as those aimed at understanding the diversification of *P. aeruginosa* populations. We have observed extensive heterogeneity in natural populations of *P. aeruginosa* recovered from CF patient sputa [18, 19]. Similar phenotypic and genotypic diversification can be observed during growth in ASM[4] (and our unpublished data), making it an attractive *in vitro* model of the CF lung conditions. The relative simplicity of the ASM model makes it easy to design long-term adaptation experiments aimed, for example, at monitoring the effects of antibiotics or other stresses on *P. aeruginosa* population divergence. In addition, other bacterial pathogens can be grown in ASM. For example, Fouhy *et al.* 2007 have used ASM to study biofilm formation by *S. maltophillia*[20].

Disclosures

No conflicts of interest declared.

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