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Testing physiologically relevant conditions in minimal inhibitory concentration assays

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The minimal inhibitory concentration (MIC) assay uses agar or broth dilution methods to measure, under defined test conditions, the lowest effective concentration of an antimicrobial agent that inhibits visible growth of a bacterium of interest. This assay is used to test the susceptibilities of bacterial isolates and of novel antimicrobial drugs, and is typically done in nutrient-rich laboratory media that have little relevance to in vivo conditions. As an extension to our original protocol on MIC assays (also published in *Nature Protocols*), here we describe the application of the MIC broth microdilution assay to test antimicrobial susceptibility in conditions that are more physiologically relevant to infections observed in the clinic. Specifically, we describe a platform that can be applied to the preparation of medium that mimics lung and wound exudate or blood conditions for the growth and susceptibility testing of bacteria, including ESKAPE pathogens. This protocol can also be applied to most physiologically relevant liquid medium and aerobic pathogens, and takes 3–4 d to complete.

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Introduction

The standardized Clinical and Laboratory Standards Institute method, which is used to measure the effects of antimicrobials against pathogens causing infections in the clinic, determines the minimal inhibitory concentrations (MIC) of antimicrobials in vitro[1,2]. Traditionally, this assay has been performed in nutrient-rich medium, e.g., Mueller Hinton broth (MHB), and can be done through agar dilution or broth dilution of the antimicrobial agent. The MIC is defined as the lowest concentration of the antimicrobial agent where no growth is observed under defined conditions. The conventional protocol is described in detail in *Nature Protocols*[1] and can be used to test the activity of antibiotics, antimicrobial peptides and other antimicrobial agents against aerobic bacterial isolates.

Although this protocol and other bacteriology studies have classically been performed in rich media such as MHB, where bacteria grow optimally and antimicrobial activities are often highest, this is not truly representative of activities in a clinical environment[3]. Indeed, the physiology of the host infection environment has been shown to alter growth, expression and essential gene patterns in various pathogens[4–10] and, likely as a consequence, influences antimicrobial susceptibility[11–13]. Of particular interest in health care settings are the well-known ESKAPE pathogens (*Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter sp.*), which are difficult to treat and have been declared some of the most critical pathogens to target for novel drug research and development by the World Health Organization[14,15].

One ESKAPE pathogen, *P. aeruginosa*, grown in conditions such as minimal medium and medium containing lung sputum of patients with cystic fibrosis (CF), has different essential genes for survival, when compared with *P. aeruginosa* grown in lysogeny broth (LB)[10,16]. Furthermore, global transcriptional analysis has determined that gene expression of organisms, including the ESKAPE pathogens, is substantially altered in human lung sputum and blood, serum, or plasma[17–19]. These in vitro and ex vivo models appear to be better mimics of the host environment than nutrient-rich laboratory media for bacterial physiological and mechanistic studies[4,20]. For example, transcriptional analysis of *P. aeruginosa* grown in Roswell Park Memorial Institute medium (RPMI) with 20%

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Table 1 | Accuracy scores (AS_2) of expression of genes in all PseudoCap[21] functional classes in RPMI +5% MHB (RPMI) and RPMI+5%MHB +20% human serum (RPMI/serum), MOPS succinate, synthetic CF sputum medium 2 (SCFM2), and LB compared with in vivo transcriptomic data from CF sputum samples and chronic wound

Functional class	AS_2 (%)[a]				
	RPMI	RPMI/ serum	MOPS succinate	SCFM2	LB
Adaptation and protection	75	75	73	88	73
Amino acid metabolism and biosynthesis	84	78	83	87	80
Antibiotic resistance and susceptibility	79	79	79	71	64
Biosynthesis of cofactors, prosthetic groups and carriers	86	84	76	81	84
Carbon compound catabolism	82	88	82	97	91
Cell division	94	94	94	94	88
Cell wall LPS capsule	87	91	91	96	89
Central intermediary metabolism	80	80	92	92	76
Chaperone and heat shock	100	100	75	94	94
Chemotaxis	89	67	78	56	78
DNA replication and repair	89	92	81	94	92
Energy metabolism	75	73	76	88	72
Fatty acid and phospholipid metabolism	90	86	81	86	86
Membrane	83	80	74	83	73
Motility	90	81	81	86	81
Nucleotide biosynthesis and metabolism	93	93	93	100	75
Protein secretion and export	93	100	73	93	87
Putative enzyme	90	88	76	96	86
Secreted factors	100	100	78	100	67
Transcriptional regulator	73	64	70	75	59
Transcription RNA processing and degradation	91	91	78	87	78
Translation and posttranslational modification	90	88	69	91	71
Transport small molecules	84	81	72	84	69
Two-component regulators	95	89	79	74	68

[a]AS_2 represents the percentage of genes within two standard deviations of the mean expression levels in CF sputum and chronic wound samples as determined with *z*-score calculations using the mean of normalized read counts. Analysis was performed by Belanger et al.[20].

human serum and 5% MHB compared with *P. aeruginosa* grown in MHB[20] found that resistome genes, from various PseudoCap[21] functional classes that are important for antibiotic susceptibility, were dysregulated in host-mimicking conditions compared with MHB alone. Comparisons of global gene expression profiles between host-mimicking models, nutrient rich-laboratory media and in vivo host conditions (such as CF lung sputum and wound exudate) can be obtained by measuring accuracy scores based on the proportion of genes with expression levels within two standard deviations (AS_2) of the in vivo target (for example, assessing gene expression directly in animals or human infections). As seen in Table 1, when using combined in vivo *P. aeruginosa* transcriptomes from CF sputum and wound exudate as the in vivo target, host-mimicking models such as synthetic CF sputum medium (SCFM), RPMI and RPMI with human serum outperform LB and nutrient-limiting medium 4-morpholinepropanesulfonic acid (MOPS)–succinate by showing expression patterns more similar to the target for genes belonging to most functional classes (see bolded numbers in Table 1). This demonstrates that physiologically relevant medium more closely resembles the human host than rich laboratory medium.

Furthermore, there is evidence that such systems might provide a better indication of potential in vivo drug efficacy in susceptibility testing[20,22]. For this reason, a shift in the way we approach susceptibility testing has led to the proposal that one should also test antimicrobial activity in conditions that are more relevant to those of clinical infections[12]. Antimicrobials have different activities against pathogens depending on the medium used, particularly in conditions that mimic the host environment[20,23–26]. For example, serum-containing tissue culture based medium has a substantially (up to 64-fold) reduced MIC for azithromycin[20,23], an unaltered MIC for ciprofloxacin[24], and an increased MIC (by 16-fold) for minocycline[24] when compared with rich laboratory medium.

For azithromycin, the validity of the lower MIC in host-mimicking medium has been confirmed by animal model experiments[20,23]. Supplementary Table 1 contains a side-by-side comparison of MICs in MHB and under physiologically relevant conditions. In 2015, Colquhoun et al. used lung surfactant from cows and human serum to test antimicrobial activities of various classes of antibiotics against *A. baumannii* and *P. aeruginosa*[24]. They discovered 19 antimicrobial agents that had activity against *A. baumannii* in nontraditional media that was not predicted in MHB. Lin et al.[23] and Belanger et al.[20] demonstrated bactericidal activity of macrolides against Gram-negative pathogens *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* under medium conditions mimicking human wound exudate or blood, even though these antibiotics are only bacteriostatic and modestly effective in MHB[20,22]. Recently, Weber et al. identified antimicrobial compounds active against *K. pneumoniae* in human serum that were previously overlooked when using traditional methods[26]. They identified 13 compounds that had at least fourfold greater activity in serum when compared with the nutrient-rich laboratory medium, LB, including the antibiotics polymyxin B, colistin, azithromycin and rifaximin as well as antimicrobial molecules 9-aminoacridine, bleomycin, mitoxantrone, floxuidine, 5-fluorouracil and carmofur[26].

Applications

Exploring the altered, especially increased, activity of antibiotics in physiologically relevant media may help researchers to find new treatments for multidrug-resistant pathogens that are difficult to treat via traditional methods and gain better insights into the practical (in vivo) susceptibility of bacteria isolated from the clinic. Here we highlight the utilization of host-mimicking conditions in the MIC assay as an extension to our previous protocol[1]. This protocol focuses on broth dilution MIC methods using liquid media designed to be more relevant, physiologically, to host conditions. The liquid medium is used to serially dilute the antimicrobial agent being tested and is then inoculated with bacteria at a known concentration. Although previously published broth macrodilution protocols can also be used with host-mimicking media, the large amounts of medium and antibiotic agents required makes broth macrodilution less feasible for testing antibiotics given the increased cost and reduced availability of materials required for host-mimicking media. Thus, this protocol will focus on microdilution methods in a 96-well format.

Using physiologically relevant medium is intended to increase the relevance of MIC protocols in the context of human infections. There are additional considerations that must be taken when performing MICs in host-mimicking conditions. These considerations are related to (i) defining an appropriate host-mimicking medium for the bacterium of interest, (ii) preparing the medium, (iii) accounting for the turbidity of the medium being used and (iv) altering timing considerations when using host-mimicking media. These will be discussed in detail in this protocol with direct reference to aspects of the standard MIC protocol[1].

Bacteria

The ESKAPE pathogens are of great interest in antimicrobial activity studies since they are of global concern in nosocomial infections due to their increased multidrug resistance to many important antibiotics commonly used in the clinic[14,15]. These opportunistic pathogens are often associated with pneumonia and chronic lung infections, soft tissue and wound infections, bacteremia and sepsis, and urinary tract infections[15]. The protocol described here is applicable to any strain/clinical isolate using assay conditions appropriate for testing the susceptibility of MDR ESKAPE pathogens that can be isolated and grown aerobically in the laboratory.

Bacteria used in MIC assays must be identified to the genus and species level and grown in pure culture prior to performing the assay. MIC assays require a standard and consistent inoculum concentration of bacteria to accurately measure the effective antimicrobial concentrations. Since correlations between bacterial optical density (OD_{600}) and colony-forming units per mL (CFU/mL) will differ between different bacteria, the correlation between OD_{600} and CFU/mL needs to be determined for the specific bacterium prior to performing the MIC assay.

Media

To produce results that are indicative of antimicrobial activity of a specific agent against a pathogen of interest, the medium used must reflect, to some extent, the environment in which the pathogen is commonly found. Host-mimicking medium typically consists of liquid medium containing

host factors, carbon and nitrogen sources, as well as other minerals typically found within a specific tissue or infection. There are multiple possible host-mimicking conditions to use depending on the type of environment and the pathogen of interest. This protocol focuses on host-mimicking conditions developed to mimic the lung, wound exudate and blood of human patients, although alternative assays are being developed to study bacteria in conditions relevant to human infection.

For example, conditions used to asses antimicrobial activity against pathogens causing pneumonia or chronic lung infection can be mimicked by using lung bovine surfactant[24] or human patient sputum[4,6], or by creating synthetic media to mimic the lung environment[4,27–30]. One such medium, called SCFM, was developed by Palmer et al.[27] to contain all of the amino acids, carbon and other nutrients that are commonly found in CF lungs, at the concentrations identified in the lung. See 'Reagent setup' for detailed methods to create this media for antibiotic susceptibility testing.

Other key components of the lung sputum of CF patients are polymeric glycoproteins called mucins as well as DNA[31,32]. Alternative and modified media mimicking the CF lung (mucin-modified SCFM, artificial sputum medium and artificial sputum medium DNA-free modified) contain between 0.4% and 1% porcine stomach mucin and between 1.4 and 4 mg/mL herring sperm DNA to mimic the turbidity and surfactants present in the lung environment[22,28–30]. Of note, while utilizing medium conditions containing mucin in an MIC assay, is that the medium will be turbid and will not allow for visual growth determination. For media such as this, plating of the inoculated serial dilution wells after incubation is required. Media mimicking lung mucus are better prepared fresh or the day before the assay, but can be stored for up to a few weeks at 4 °C. Mucin content in the medium will settle and needs to be well mixed prior to setting up the assay.

Human blood, human serum and tissue culture media with or without human serum are other in vitro conditions that have been used to mimic wound exudate or blood[7,23–26]. It is worth noting that not all bacterial species can grow on whole human blood, but most tolerate diluted blood or serum. Conditions that can be used to mimic these environments include, but are not limited to, whole human blood[25,26], human serum[24] and RPMI-1640 ± 20% human serum +5% MHB (% represents vol/vol)[20,23]. Human blood can be collected freshly from available donors or purchased from supplier companies. Tissue culture media with MHB and any media containing blood or serum should be prepared fresh the day of the MIC assay. Conditions that include serum and tissue culture media are excellent for use in MIC assays since they remain transparent and allow for visual determination of growth. Medium containing whole blood will need to be plated to determine growth. See 'Reagent setup' for detailed methods for preparation of RPMI-1640+20% human serum +5% MHB.

Limitations and alternative protocols

Similar to traditional MIC assays, observing the MIC of an antimicrobial agent using visual or optical density measures does not differentiate between bactericidal and bacteriostatic activity. Plating wells of the MIC assay must be done to determine the minimal bactericidal concentrations and to determine MICs in turbid media.

This protocol describes a method for aerobic growth at 37 °C; however, it should also be appropriate under microaerophilic conditions. Furthermore, this protocol focuses on published physiologically relevant conditions that have been used to study ESKAPE pathogens. Other host-mimicking media used to culture various bacterial isolates continue to be studied and may also be appropriate for susceptibility testing.

When broth dilution methods are used with peptides, which can aggregate at high concentrations in media or bind to polystyrene plastics used in microdilution assays, dilutions should be performed in polypropylene plates with 10 μL of water, then inoculated by adding, to the prediluted peptides, bacteria suspended in the equivalent physiologically relevant medium.

Additionally, antibiotics that are more effective in host-mimicking conditions, or bacteria that have impaired growth in host-mimicking conditions, may take longer than 24 h to enable visual confirmation of the concentration of antimicrobial required to inhibit the pathogen. Furthermore, host-mimicking conditions often encourage alterations in growth states of bacterial cultures such as biofilm formation (i.e., growth of bacteria adherent to the walls of testing wells). Alternative methods to classical MIC assessments are used to measure biofilm inhibition or biofilm eradication and can be performed under the same host-mimicking conditions[33].

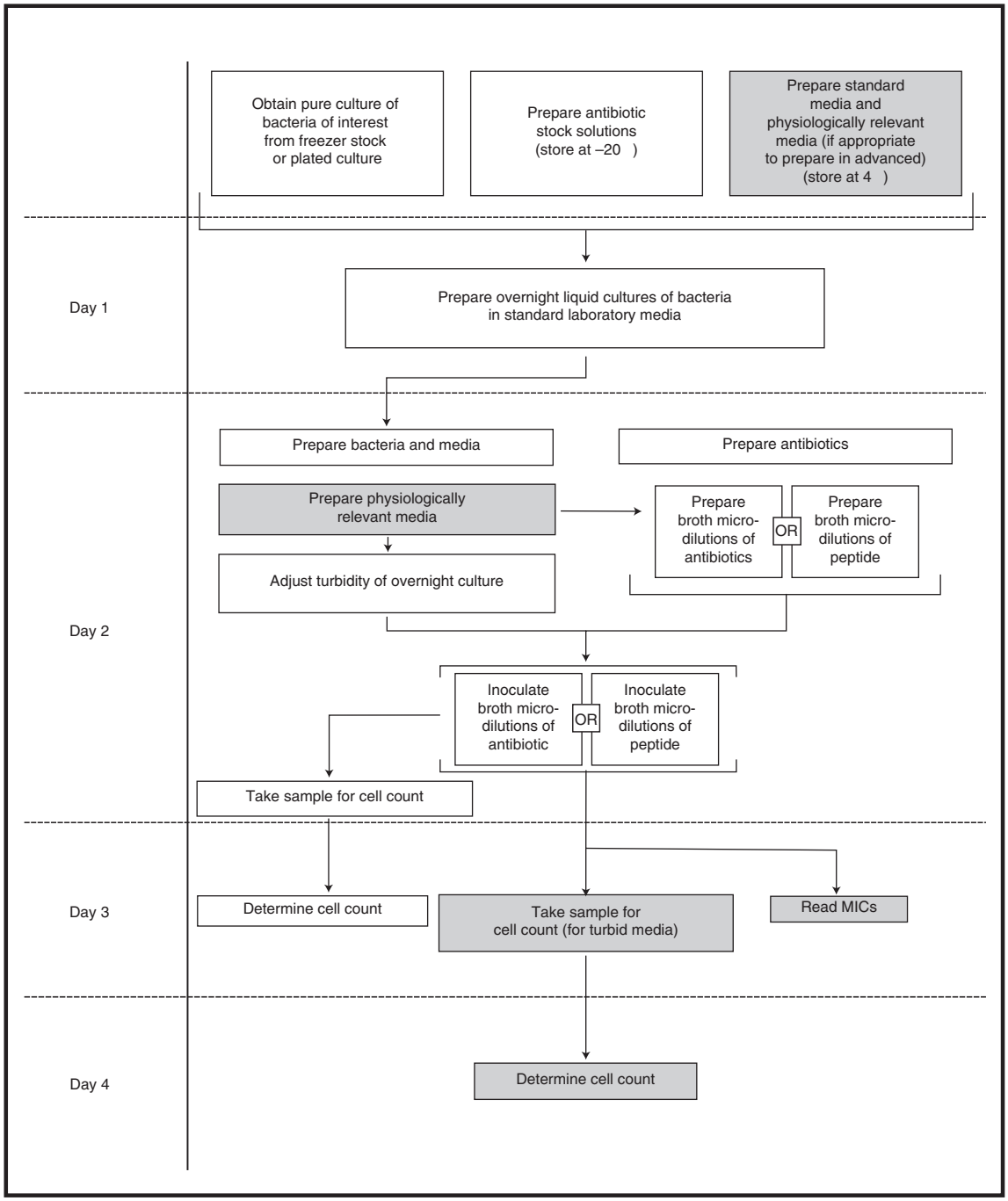

Fig. 1 | Simplified schematic of broth microdilution MIC assay using host-mimicking media. Steps requiring additional considerations to traditional previously published MICs are shaded in gray.

Experimental design

For performing MICs in host-mimicking conditions, additional time is required and certain design considerations come into play relative to the standard MIC protocol[1]. The type of medium and its properties must be considered prior to designing the MIC experiment, since this will impact on the medium preparation and method of growth determination used. A simple flow diagram of the protocol in Fig. 1 demonstrates the steps required to perform MICs in microdilution assays with physiologically relevant media. Particularly crucial steps for the extended protocol are shaded in gray.

Materials

Equipment

! CAUTION Appropriate laboratory containment and biosafety protocols should be followed depending on the biosafety level of the bacteria being studied. Use aseptic technique during all bacterial growth steps to avoid possible contamination.

The equipment listed below is the same as that used for the standard protocol[1] with the inclusion of additional equipment required for preparing physiologically relevant media.

- Spectrophotometer suitable for measuring at wavelengths of 600 and 625 nm (Eppendorf, cat no. 6135 000 009)
- Microplate reader capable of measuring optical density of 96-well plates at 600 nm (BioTek, Synergy H1)
- For antibiotics: sterile 96-well polystyrene microtiter plates, BD Falcon (Thermo Fisher Scientific, cat. no. 351177)
- Magnetic stirrer/hotplate for mixing of medium components (Thermo Fisher Scientific, cat. no. SP88857108)
- For cationic antimicrobial agents such as peptides: 96-well polypropylene microtiter plates (Costar, cat. no. 3879) **▲ CRITICAL** Avoid tissue-culture-treated or polystyrene plates as these are strongly negatively charged and will nonspecifically bind peptides.
- 1.5 mL Eppendorf microcentrifuge tubes (Thermo Fisher Scientific, cat. no. 14-222-168), sterilized
- Shaker, suitable for test tubes 13 × 100 mm (Thermo Fisher Scientific, cat. no. SHKE4000-8CE)
- Glass tubes, 13 × 100 mm, sterilized (Thermo Fisher Scientific, cat. no. 99445-13) with sterile, autoclavable caps (Thomas Scientific, cat. no. 1198R08)
- Erlenmeyer flasks (Cole-Parmer, cat. no. UZ-34502-58)
- Sterile Petri dishes, 15 × 100 mm, Fisherbrand (Thermo Fisher Scientific, cat. no. FB0875712)
- 0.2 μm sterile cellulose acetate filters, Nalgene (Thermo Fisher Scientific, cat. no. 723-2520)
- Cell spreader, Fisherbrand (Thermo Fisher Scientific, cat. no. 08-100-11)
- Inoculation loop (Cole-Parmer, cat. no. UZ-01850-10)
- Vortex mixer (Cole-Parmer, cat. no. UZ-04724-00)
- 50 mL or 15 mL Falcon conical tubes (Thermo Fisher Scientific, cat. no. 14-432-22 (50 mL), 14-959-53A (15 mL))

For blood or serum collection

- Vacutainer rapid serum tubes for collection of serum from donors, BD Vacutainer (Thermo Fisher Scientific, 02-685A)
- Vacutainer SPS tubes, heparin tubes or buffered sodium citrate tubes for collection of whole blood or plasma from donors, BD Vacutainer (Thermo Fisher Scientific, cat. no. 02-684-19 (SPS), 02-689-6 (heparin), 02-688-26 (citrate))
- Water bath if heat inactivation of complement is desired (Thermo Fisher Scientific, cat. no. TSGP10)

For physiologically relevant medium sterilization

- 1 L sterile glass bottles autoclaved with magnetic stir bars (Cole-Parmer, cat. no. UZ-34503-41)
- Rapid-flow bottle top filters, 0.2 μm, Nalgene (Thermo Fisher Scientific, cat. no. 291-4520)

Reagents

The reagents used for MICs in host-mimicking media are similar to those described in the standard MIC protocol[1]. In this extension of that protocol, the host-mimicking medium reagents and additional reagents that differ from the original protocol are included.

- MHB, Difco (BD Diagnostics, cat. no. 275730), sterilized by autoclaving
- LB (Fisher Bioreagents, cat. no. BP1427)
- Agar, technical, Difco (BD Diagnostics, cat. no. 281230)
- Physiological saline [0.9% (wt./vol) NaCl] sterilized by autoclaving
- Sterile deionized H_2O

For media mimicking CF lung sputum

Specific reagents depend on the lung-mimicking medium of interest (e.g., SCFM, mucin-modified SCFM, artificial sputum medium and artificial sputum medium DNA-free modified). This protocol

lists the materials and methods for Palmer et al.'s SCFM medium. See recipes previously published for other detailed reagent lists[24,27–30]

For buffered base
- Sodium phosphate monobasic (NaH_2PO_4; Sigma Aldrich, cat no. S0751)
- Di-sodium hydrogen phosphate (Na_2HPO_4; Sigma Aldrich, cat no.1065860500)
- Potassium nitrate (KNO_3; Sigma Aldrich, cat. no. P8394)
- Potassium sulfate (K_2SO_4; Sigma Aldrich, cat. no. P0772)
- Ammonium chloride (NH_4Cl; Sigma Aldrich, cat. no. 254134)
- Potassium chloride (KCl; Sigma Aldrich, cat no. P4504)
- Sodium chloride (NaCl; Sigma Aldrich, cat. no. S9625)
- MOPS (Thermo Fisher Scientific, cat. no. BP308)
- Deionized water

Amino acids
- L-aspartate (Sigma Aldrich, cat. no. A6683)
- L-threonine (Sigma Aldrich, cat. no. T8625)
- L-serine (Sigma Aldrich, cat. no. S4500)
- L-glutamate·HCl (Sigma Aldrich, cat. no. G 2128)
- L-proline (Sigma Aldrich, cat. no. P0380)
- L-glycine (Sigma Aldrich, cat. no. G7126)
- L-alanine (Sigma Aldrich, cat. no. A7627)
- L-cysteine·HCl (Sigma Aldrich, cat. no. C1276)
- L-valine (Sigma Aldrich, cat. no. V0500)
- L-methionine (Sigma Aldrich, cat. no. M9625)
- L-isoleucine (Sigma Aldrich, cat. no. I2752)
- L-leucine (Sigma Aldrich, cat. no. L8000)
- L-tyrosine (Sigma Aldrich, cat. no. T3754)
- L-phenylalanine (Sigma Aldrich, cat. no. P2126)
- L-ornithine·HCl (Sigma Aldrich, cat. no. O2375)
- L-lysine·HCl (Sigma Aldrich, cat. no. W384712)
- L-histidine·HCl (Sigma Aldrich, cat. no. H8000)
- L-tryptophan (Sigma Aldrich, cat. no. T0254)
- L-arginine·HCl (Sigma Aldrich, cat. no. A5131)

Additional reagents
- Calcium chloride (CaCl; Thermo Fisher Scientific, cat. no. C69)
- Magnesium chloride (MgCl; Sigma Aldrich, cat. no. M8266)
- Iron sulfate (FeSO4•7H2O; Sigma Aldrich, cat. no. F7002)
- Glucose (Thermo Fisher Scientific, cat. no. D16)
- L-lactate (Sigma Aldrich, cat. no. L7022)

Optional additives
- Porcine mucin (Sigma Aldrich, cat. no. M2378)
- Salmon sperm DNA (Sigma Aldrich, cat. no. D3159)

For media mimicking wound exudate or sepsis
- RPMI-1640 with HEPES and L-glutamine, Hyclone (Thermo Fisher Scientific, cat. no. SH30255FS)
- Human blood or serum collected from anonymous donors or purchased commercially (Sigma, cat. no. H4522)
- MHB, Difco (BD Diagnostics, cat. no. 275730)

Reagent setup

Stock solution of antimicrobial agents

Most antibiotics in their powder form should be stored at 4 °C, unless specific storage instructions for the antibiotic are different, and the duration and conditions of storage should follow the manufacturer's instructions. Antimicrobial peptides should be stored at −20 °C. General antibiotic and antimicrobial stocks can be made and stored at −20 °C in small aliquots in volume sufficient to that required for one test or experiment. Prior to setting up MIC assays, antibiotic stocks with desired

concentrations at 2 times the highest concentration in the assay for microdilution (see Step 15A of the procedure) and 20 times the highest concentration in modified microdilution assays (see Step 15B) can be prepared and stored on ice or at 4 °C for the duration of the experiment. Weigh the antimicrobial agents using sterile containers and a spatula, taking into account the potency (μg per mg powder). The desired amount of antibiotic to weigh can be calculated as follows:

$$W = \frac{(C \times V)}{P}$$

where W = the weight of the antimicrobial agent (mg); V = the desired volume (mL); C = the final concentration of stock solution (μg/mL); and P = potency given by the manufacturer (μg/mg). Antibiotics should be dissolved in sterile distilled water or the recommended solvent. Afterwards the solutions can be filter sterilized with a 0.2 μm pore cellulose-acetate filter. Note that antimicrobial peptides should not be filter sterilized since they tend to bind to anionic surfaces such as cellulose acetate. Instead, use sterile deionized H_2O to prepare peptide stocks. For microdilutions of antimicrobial agents, always use a freshly prepared antibiotic stock or stored general stock that has not previously been thawed. Do not refreeze thawed stock solutions as this can lead to degradation of some antimicrobials such as β-lactams[2]. ▲ **CRITICAL** All host-mimicking media must be prepared using sterilized reagents and sterile technique so as to avoid contamination of the assay. Many components are not appropriate to be autoclaved and thus should be prepared by filter sterilization or in sterile H_2O. Bottles used for preparing media should be autoclaved with magnetic stir bars to allow reagents to be mixed while remaining sterile.

Lung-mimicking media

Preparation depends on the type of medium being used, and protocols for preparing these media have been published previously[27,29,30,32]. To prepare 1 L of SCFM medium as made by Palmer et al.[27], first prepare buffered salts base by adding the following to 750 mL of deionized water: 6.5 mL 0.2 M NaH_2PO_4, 6.25 mL 0.2 M Na_2HPO_4, 0.348 mL 1 M KNO_3, 1.084 mL 0.25 M K_2SO_4, 0.122 g NH_4Cl, 1.114 g KCl, 3.03 g NaCl and 10 mM MOPS. This base should be sterilized by autoclaving. Prepare all amino acid stocks except for tyrosine, aspartate and tryptophan in water at 100 mM. Make 100 mM tyrosine in 1 M NaOH, 100 mM aspartate in 0.5 M NaOH, and 100 mM tryptophan in 0.2 M NaOH. Filter the amino acids using Nalgene 0.2 μm filters. Add amino acids to the buffered base as presented in the table below, which specifies the volume of each stock solution to be added to the salts base in SCFM.

Amino acid	Volume of 100 mM stock (mL)
L-aspartate	8.27
L-threonine	10.72
L-serine	14.46
L-glutamate·HCl	15.49
L-proline	16.61
L-glycine	12.03
L-alanine	17.8
L-cysteine·HCl	1.6
L-valine	11.17
L-methionine	6.33
L-isoleucine	11.2
L-leucine	16.09
L-tyrosine	8.02
L-phenylalanine	5.3
L-ornithine·HCl	6.76
L-lysine·HCl	21.28
L-histidine·HCl	5.19
L-tryptophan	0.13
L-arginine·HCl	3.06

Additional reagents are prepared and sterilized, then added to the medium as follows: 1.75 mL of 1 M $CaCl_2$, 0.606 mL of $MgCl_2$, 1 mL of 3.6 M $FeSO_4 \cdot 7H_2O$, 3 mL of 1 M glucose and 9.3 mL of 1 M L-lactate (pH 7.0).

To make a modified SCFM medium, you can add 0.4% (w/v) porcine stomach mucin and/or between 1.4 and 4 mg/mL final concentration of herring sperm DNA. These can be added directly to the salts base or the medium and require between 1 h and overnight to go into solution. ▲ CRITICAL If adding mucin and DNA, these reagents may require a long time to go into solution and should be left stirring overnight using a magnetic stirrer prior to configuring the rest of the medium.

The final volume can be adjusted with ~5 mL of sterile H_2O. The final pH should be ~7 depending on the recipe and can be measured using an aliquot of the medium that is removed to keep the medium sterile. This medium can be stored at 4 °C for up to 1 month.

Media mimicking wound exudate or blood

Collect blood from consenting donors under institutional ethics approval in vacutainer tubes with either anticoagulant for whole blood or thrombin for serum isolation. If you do not have human ethics approval, you should purchase blood from a local supplier. Immediately after drawing blood, invert collection tubes five times to mix with anticoagulant or clotting agent. Date and anonymous donor ID should be recorded for all blood samples collected. For collection of whole blood, store at 4 °C for immediate use or freeze for long-term storage. For collection of serum, allow blood to clot in collection tubes for at least 5 min (do not let sit for >2 h before centrifugation). Centrifuge at 1,500–2,000*g* for 10 min. Heat inactivation of complement in serum is done by submerging serum in a 56 °C water bath for 30 min. Store at 4 °C for immediate use, or freeze at −20 °C for long-term storage. We recommend preparing aliquots of blood and/or serum into small volumes (5 mL) in 15 mL sterile Falcon tubes so that each aliquot is only used once.

To make 100 mL RPMI/serum medium[20], 20 mL of human serum is added to 75 mL RPMI using sterile technique. This medium should be prepared fresh on the day of use. Then, add 5 mL of sterile MHB, which can be prepared beforehand using the manufacturers' instructions and sterilized by autoclaving.

Procedure

Correlating CFU/mL with optical density of bacterial cultures ● Timing 3 d

▲ CRITICAL Correlation between CFU/mL and OD_{600} of an overnight culture should be determined for every isolate since overgrown overnight cultures may have fewer viable cells than freshly grown cultures. This should be done prior to MIC experiments. This correlation will differ between species and strains of bacteria and has to be determined for each strain being used in the MIC.

1 Streak each bacterial isolate under investigation onto an LB agar plate, and grow overnight at 37 °C.
2 Inoculate 3 mL of nutrient-rich medium MHB or LB in a sterile glass tube with a cap, using a single isolated colony from an overnight agar plate.
3 Incubate the tubes overnight in a shaker at 225 rpm at 37 °C.
4 Measure the OD_{600} of 1:10 dilutions of the liquid overnights using sterile technique (or an appropriate dilution to get an OD_{600} below 1.0; overnight cultures in nutrient-rich media will usually be between OD_{600} 1 and 3 depending on the bacterium, so a 1:10 dilution should be appropriate).
5 Dilute the overnight culture 1:100 using sterile tubes and nutrient-rich broth or sterile saline.
6 Dilute this 1:100 solution from Step 5, five times in a 1:10 dilution of 100 µL to obtain final dilutions from 10^{-3} to 10^{-7}. This can be done in wells of a 96-well plate for multiple strains or replicates at once.
7 Spread plate the last four dilutions (10^{-4} to 10^{-7}; 10 µL of each dilution on a marked quarter of a Petri plate) onto LB agar plates using a sterile cell spreader. Spread the plated volume evenly starting with the most diluted (10^{-7}) culture and working your way to the least diluted culture.
8 Incubate the plates overnight at 37 °C.
9 Count the numbers of colonies on the plates (only considering quadrants with fewer than 300 colonies).
10 Calculate CFU/mL in the overnight culture using the following formula:

$$N = C \times 10^{D} \times 100$$

where N = CFU/mL; C = number of colonies; D = dilution factor (e.g., for a $1{:}10^7$ dilution $D = 7$).
11 Calculate the average of the CFU/mL between dilutions with <300 colonies, and correlate this number with the OD_{600} of the overnight culture. This will be consistent for the same bacterium grown under the same conditions in future experiments. Plating 10 µL of 10^{-4} to 10^{-7} dilutions will

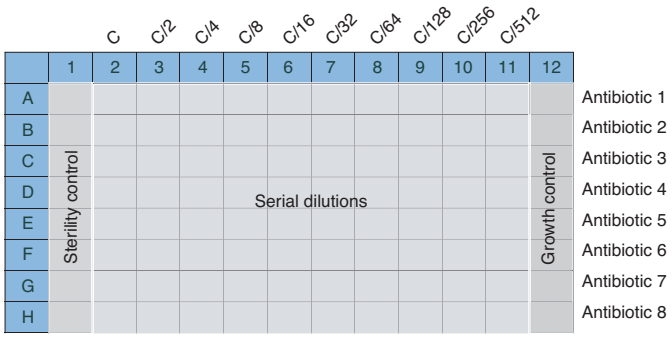

Fig. 2 | Outline of a 96-well plate MIC assay with eight different antibiotics. C indicates the highest concentration tested with twofold serial dilutions in each adjacent column of the plate from left to right. Sterility control on the far-left column of the plate (column 1) contains broth only, and growth control on the far-right column of the plate (column 12) contains broth inoculated with bacteria without antibiotic.

allow for a limit of quantitation as low as 10^6 CFU, which should be equivalent to an approximate OD_{600} of 0.001 for most *E. coli* or *P. aeruginosa* strains.

PAUSE POINT The correlation between CFU/mL and OD_{600} does not need to be performed at the same time of the MIC experiment. As long as the correlation has been determined for the bacterial species of interest, the MIC can be determined for the bacterium at any time.

Preparing reagents

Preparation of bacterial cultures ● Timing 5 min/overnight incubation/5 min

12 Streak each bacterial isolate under investigation on an LB agar plate, and grow overnight at 37 °C.

13 Prepare cultures for inoculation in a standard laboratory medium, using one of the following three options (described previously in more detail)[1]:

- Directly suspend the colonies from agar plates into broth, then immediately measure for turbidity and use to inoculate the assay.
- Use multiple isolated colonies to start a broth culture in glass tubes, which will reach appropriate turbidity in between 3 and 6 h of incubation.
- Set up overnight cultures by inoculating broth with a single isolated colony, after which the overnight cultures will be measured for turbidity and used to inoculate the assay.

▲CRITICAL STEP Using standard laboratory media such as LB or MHB at this step of the MIC ensures the measurement of optical density is accurate according to standardized methods and allows for correct inoculum density calculations.

Preparation of physiologically relevant media ● Timing ~30 min to 1 h

14 Prepare media by following the detailed instructions in the 'Reagent setup' section. Lung-mimicking media can be prepared ahead and stored for up to 1 month, whereas blood or serum-containing media should be prepared fresh on the day. On the day of the assay, blood or serum components stored at −20 °C can be thawed on ice while bacterial cultures are prepared.

!CAUTION Before collecting sputum, surfactant or blood samples from human donors, ensure the donors have signed the informed consent for donating and that all required ethics are in place.

▲CRITICAL STEP If preparing lung-mimicking media in advance, they should be checked for contamination visually or by plating on nutrient agar (if medium is visually turbid) and mixed well to ensure components such as mucin are homogeneously distributed.

Antimicrobial testing ● Timing 2 d

15 Prepare the antimicrobial agent in liquid physiologically relevant medium using a broth microdilution method (Fig. 2). Antimicrobial dilution series can either be performed as microdilutions in host-mimicking medium in polystyrene 96-well plates (option A) or modified microdilutions in water in polypropylene plates (option B), depending on the medium and tendency of the antimicrobial agent to bind to polystyrene plates or aggregate at high concentrations in medium. For both of these methods, the first and last column of the 96-well plate should be kept as a negative sterility control containing medium with no antibiotic or bacteria

and a positive growth control with bacteria added but no antibiotic treatment, respectively. Antimicrobial treatment can be diluted in columns 2–11 of the 96-well plate. A minimum of three repeats for each antibiotic against each bacterium of interest should be tested using three separate biological replicates of the bacterial cultures. We recommend these be repeated on separate plates over multiple days. More replicates are required when results disagree between replicates.

(A) **Microdilutions ● Timing 2 d**

(i) Prepare the antibiotic stock(s) (see 'Reagent setup') to two times the highest desired concentration in the dilution series in a solvent that is appropriate for the antimicrobial(s) of interest.

(ii) In 96-well format, add 90 µL of host-mimicking medium to each well.
▲ **CRITICAL** Keep the lid of the 96-well plate when not handling to avoid contamination of the MIC assay.

(iii) Add 90 µL of antibiotic to the first lane of the plate containing 90 µL of medium (column C in Fig. 2), mix well (1:2 dilution) and then transfer 90 µL to the next well. This is repeated down the lanes of the plate, leaving one lane for growth control and one lane for a negative sterility control.
▲ **CRITICAL STEP** It is important to have negative control wells containing host-mimicking media without bacteria on every 96-well assay plate. These will act as sterility controls, but also, since different media will have different OD_{600} values, they are visual references of what growth-inhibited wells should look like after incubation. After the dilution series, each well will contain a final volume of 90 µL.

(iv) Check that the final bacterial concentration is between 10^5 and 10^6 CFU/mL. A bacterial concentration of 5×10^5 CFU/mL can be reached by adding 10 µL bacterial suspension (adjusted to 5×10^6 CFU/mL from Step 13) to 90 µL of host-mimicking medium containing the microdilution of antibiotic.

(v) Plate inoculum and control wells for CFU counts by taking 10 µL of each of the inoculum, inoculated well and sterility control well and suspending in 90 µL of nutrient-rich mediium or PBS in a separate 96-well plate (1:10 dilution).

(vi) Mix well, and repeat serial 1:10 dilutions three more times in adjacent wells until you have a final 10^{-4} dilution. Spread plate 10 µL of all four dilutions on four quadrants of a labeled LB agar plate.

(vii) Incubate 96-well assay plates and CFU count plates for 16–20 h statically at 37 °C.

(viii) Count colonies on plates for each dilution, and proceed to Step 16 for determining MIC result.

(B) **Modified microdilutions for cationic peptides or aggregating antibiotics ● Timing 2 d**
▲ **CRITICAL** The stock concentration of antimicrobial has to be prepared at 20 times the highest desired concentration in the dilution series.

(i) First, add 10 µL of water to all wells of a 96-well polypropylene plate. Then, add 10 µL of antimicrobial to the first lane of wells containing water (column C in Fig. 2; 1:2 dilution), and mix well. Next, transfer 10 µL to the following well, continuing the twofold dilution series through the remainder of the plate (except for the growth and sterility controls).

(ii) Check the final bacterial concentration is between 10^5 and 10^6 CFU/mL. A bacterial concentration of 5×10^5 CFU/mL is reached by adding 90 µL of bacterial suspension (5.6×10^5 CFU/mL adjusted from Step 13) in host-mimicking medium to 10 µL of water containing antibiotic or antimicrobial peptide serial dilution.

(iii) Follow CFU plating and incubation steps from Step 15A(v–viii).

Determining the MIC ● Timing 10–20 min (with overnight culture for option B)

16 Determine the MICs using one of the following methods, depending on the medium and antimicrobial susceptibility testing method used.

- For microdilutions in polystyrene plates using medium that is not turbid (i.e., not containing mucin or whole blood), 96-well plates can be read for OD_{600} on a plate reader. Growth can also be visibly determined or determined with plating
- For turbid media and modified microdilutions in polypropylene plates, measuring OD_{600} will not be a viable way of determining growth. Instead these assays will have to be plated for growth (see Steps 15A(v–viii)). This will require an additional day to determine bactericidal concentrations of the antimicrobial agents. Plating 10^{-2} to 10^{-7} dilutions would allow for a limit of quantitation between 10^4 and 10^{11} CFU/mL. This method effectively determines concentrations of antibiotic

that inhibit bacterial growth. To determine bactericidal concentrations of antibiotic, undiluted aliquots from treated wells must be plated

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
12–15	Microbial growth in sterile control	Broth was contaminated	Use sterile broth, and prepare fresh host-mimicking media
		Pipette tips were not sterile	Use sterile pipette tips
		Tubes used for preparing stocks were not sterile	Ensure tubes used for making solutions are sterile. Can autoclave to be sure
		Contamination from bacteria added to adjacent wells in 96-well format	Pipette carefully to avoid spilling over bacteria or making aerosols
14	No growth observed in overnight cultures of bacteria in host-mimicking medium	Bacteria being used does not grow in host-mimicking medium being used or grows very slowly	Try using a different host-mimicking medium, supplement with larger amounts of nutrient medium or increase the growth time
	Low turbidity and growth observed in overnight cultures of bacteria in host-mimicking medium	Restricted nutrient conditions could limit growth rate	See solution above, but particularly increase the growth time
	Bacteria do not grow well in host-mimicking medium but attach to walls or surface (pellicle) of growth chamber	Restricted nutrient conditions could preferentially induce biofilm formation	Test biofilm growth using an alternative protocol, e.g., by measuring crystal violet staining of biofilm[33]
	Growth in negative controls with blood or serum-containing media	Contamination in blood or serum sample	Filter sterilize serum using a 0.2 μm filter (which also removes blood cells)
14 or 15	No growth in growth control wells of assay	Inoculum was too low. Growth condition was not appropriate for the microbe being grown (see additional growth problems with host-mimicking media above)	Determine cell count of bacterial suspension for all bacterial isolates used in the assay

Timing

Steps 1–11, correlating CFU/mL with optical density of bacterial cultures: 3 d
Steps 12–13, preparing bacterial cultures: 5 min/overnight incubation/5 min
Step 14, preparing host-mimicking media ~30 min to 1 h
Step 15, microdiluton and modified microdilution plate setup: 2 d
Step 16, determining the MIC: 10–20 min (with overnight incubation for option B)

Anticipated results

For assays to be valid, agar plates for cell count in Step 5 must be checked for the correct number of CFU. Colony counts of the inoculum should agree with measured optical density values when setting up the assay. Positive control wells should contain between 10^5 and 10^6 CFU/mL. Agar plates with dilutions of the negative control wells should not have any growth. In an assay with a starting bacterial concentration of 5×10^5 CFU/mL, the day after plating, the agar plate for the inoculum of antibiotic microdilution assays should contain ~50 colonies on the $1{:}10^3$ dilution quadrant and ~5 colonies on the 1:10,000 dilution. The inoculum plate for cationic peptide microdilution assays should contain ~45 colonies on the 1:100 dilution quadrant and 4–5 colonies on the 1:1,000 dilution quadrant. The growth control wells for each assay should have ~45 colonies on the 1:100 dilution quadrant and 4–5 colonies on the $1{:}10^3$ dilution quadrant. Agar plates with dilutions of negative control wells should not have any growth.

After incubation, the growth control should be visibly turbid, or, if the medium is turbid, then the agar plates for positive growth control wells should have growth by the following day. Negative

control wells should not have any growth after incubation. If there is growth in the negative (sterility) control, the MIC values are not valid. The MIC is defined as the lowest concentration of antimicrobial agent that inhibits visible growth of the bacterium being tested. In medium that is turbid, only a bactericidal and not a bacteriostatic concentration can be determined, as plating is required to measure growth in each of the wells. Supplementary Table 1 provides some sample MIC data, but actual numbers will vary from laboratory to laboratory and between test strains.

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Competing interests

The authors declare no competing interests.

Additional information

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