Assessing the Airborne Survival of Bacteria in Populations of Aerosol Droplets with a Novel Technology

Supplementary Information

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1. BACTERIAL STRAINS AND CULTURE

The Luria-Bertani (LB) broth was prepared by dissolving 5 g of yeast extract (Sigma-Aldrich Ltd., UK), 10 g of NaCl (Fisher Scientific, UK) and 10 g of Tryptone (VWR International Ltd, UK) in 1000 mL of sterilized deionized (DI) water and then sterilizing the mixture in an autoclave (Classic, Prestige Medical, UK). Luria agar was prepared by adding 20g L^{-1} of Granulated Agar (BD Difco™ Dehydrated Culture Media, Fisher Scientific, UK) to the previous mixture before sterilization.

The microorganisms used to evaluate this methodology were *Escherichia coli* MRE162 and spores of *Bacillus atrophaeus.* Both strains were obtained from the in-house culture collection

at the Defence Science & Technology Laboratories (Dstl, Porton Down, Salisbury, United Kingdom).

E. coli MRE162 was cultured on LB agar plates at 37℃ for 24 h for the isolation of single colonies. Stock cultures were maintained at -20℃ in LB broth containing 20% (w/w) glycerol with a microbial concentration of $1.4 \pm 0.2 \times 10^9$ (mean ± standard deviation) CFU ml^{-1}. For aerosol exposures, bacterial suspensions were prepared by culturing 2 µL of a stock culture inoculated in 200 mL of LB broth and shaken at 180 rpm for 24 h at 37 ℃, producing a concentration of $2.7 \pm 1.7 \times 10^9$ CFU ml^{-1} in the stationary phase. A ten-fold serial dilution from this culture was prepared in LB broth for aerosolization. A *B. atrophaeus* stock culture with a microbial concentration of $6.5 \pm 0.3 \times 10^9$ spores ml^{-1} (triple washed in distilled water) was prepared for aerosolization by diluting 50µL of an original water stock in 450 µL of Phosphate-Buffered Saline (PBS).

2. SAMPLE PREPARATION FOR DETERMINATION OF MICROBIAL CONCENTRATION IN INDIVIDUAL BIOAEROSOL

Yellow-green fluorospheres beads (1 µm diameter; Molecular Probes, United Kingdom) with a commercial concentration of 3.6×10^{10} beads mL^{-1} were used as a surrogate of bacterial cells. Sequential dilutions were prepared in LB broth creating fluorosphere suspensions with concentrations down to 3.6×10^6 beads mL^{-1}. To cover a similar range of concentration, an E. *coli* MRE612 culture in the stationary phase was concentrated by centrifugation of 4 mL of culture at 2000 RCF for 5 minutes and resuspended in 1 mL of fresh LB broth to a cell suspension of 9.3×10^9 CFU mL^{-1} following staining with SYTO-9 (Molecular Probes, United Kingdom). Ten dilutions were prepared in LB broth with concentrations between 9.3×10^5 and of 9.3×10^9 CFU mL^{-1}. A stock of *B. atrophaeus* spores in water with concentration of 4.0×10^9

CFU mL^{-1} was stained with fluorescein isothiocyanate (FITC, Sigma-Aldrich Ltd., UK) and five different dilutions were prepared in water from this stock down to a concentration of 3.0×10^5 CFU mL^{-1}.

3. STAINING PROTOCOLS FOR BACTERIAL VIABILITY AND COUNTING

For the evaluation of membrane integrity, samples containing *E. coli* MRE162 or *B. atrophaeus* spores were subjected to different staining processes to determine the physiological condition of bacteria cells after aerosolization and the number of cells enclosed within the bioaerosol droplets.

Live/Dead BacLight bacterial viability and counting kit (Molecular Probes, United Kingdom) was used to determine the aerosolization effect on bacterial viability as measured by a decrease in observed cellular SYTO-9 signal. Bacteria with intact cell membranes fluoresce bright green (SYTO-9) while bacteria with compromised cell membranes fluoresce red (propidium iodide [PI]). For 'live' and 'dead' controls, *E. coli* cultures were either untreated (not aerosolised) or treated with 75% (vol/vol) ethanol for one hour at room temperature, respectively. Staining procedures for the samples were performed immediately after collection following the manufacturer's protocol. Specifically, bacterial cells were stained by adding 3 µL of a 10^{-3} dilution of a 1:1 mixture of 8 µL of 3.34 mM SYTO9 with 8 µL of 20 mM PI from the Live/Dead BacLight bacterial viability kit to the 20 µL samples. Subsequently, samples were placed on microscope slides coated with 5% (vol/vol) porcine gelatine (Sigma-Aldrich Ltd., UK) for microscopic and image analysis.

SYTO-9 (Molecular Probes, United Kingdom) and FITC (Sigma-Aldrich Ltd., UK) dyes were used to determine the concentration of bacteria and spores in bioaerosol particles generated with a DoD, respectively. *E. coli* MRE162 bacteria were labelled by adding 4 µL of 3.34mM

SYTO9 to 1mL of a four-times concentrated bacterial culture for 24h at 4°C. *B. atrophaeus* spores were treated with 2.56 µM FITC for 24h at 4°C. In addition, 1% (vol/vol) of aqueous Tween 80 was added at to give a final concentration of 0.1% (vol/vol) to promote the separation of cells in all samples. For samples in the order of 10^7 CFU ml^{-1} or lower, 1% (vol/vol) of a FITC water solution with 0.5% (vol/vol) concentration was added to enhance the visibility of the aerosol droplets under the confocal microscope. Aerosolised particles were collected on to coated microscope slides for microscopic and image analyses.

4. MICROSCOPIC ANALYSIS OF STAINED CELLS

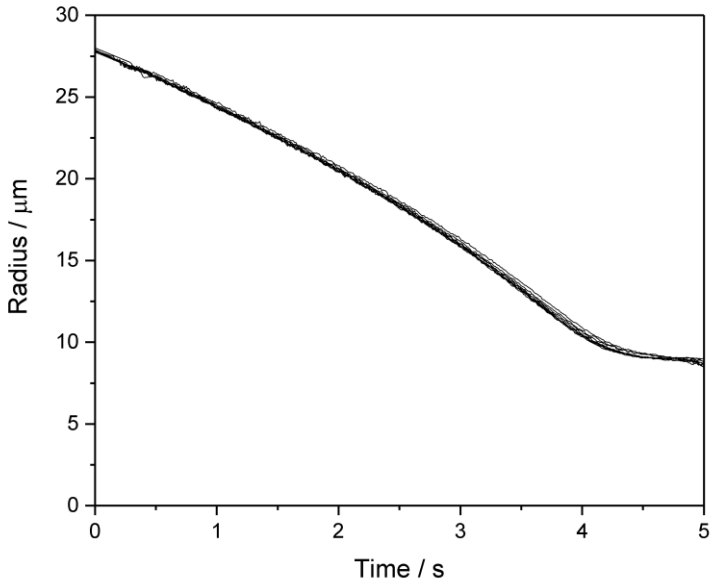
All samples were analysed with a dual mode (confocal/widefield) imaging system at the Wolfson Bioimaging Facility, University of Bristol (SPE single channel confocal laser scanning microscope attached to a DMi8 inverted epifluorescence microscope, Leica Microsystems, Germany). Fluorescent samples were excited by the 488nm-spectral line and detected using the green and red channels (590nm LP, 425nm LP). ImageJ software [1] was used to process all images acquired with the confocal/widefield system for both the determination of bacterial viability (proportions of cells exhibiting green fluorescence) after aerosolization and the enumeration of particles encapsulated within the aerosol droplets.

5. SIZE DISTRIBUTION OF DROPLETS AND TIME DEPENDENCE OF CHANGE IN SIZE

During levitation, the droplets decrease in size by losing water until they reach an equilibrium size of ~5 µm radius, depending on the relative humidity in the cell. Measurements of the initial size distribution, the kinetics of water loss and the equilibrium size are made using the Comparative Kinetics Electrodynamic Balance (CK-EDB) system, described in detail in our

previous publications.[2] Figure S1 shows an example of the variability in the initial droplet size from droplets generated from LB containing *E. coli* cells with a concentration of 6.6710^8 cells mL^{-1} at 33.2% RH, showing an initial droplet size of 27.8±0.08 μm.

Figure S1: Measurement of the initial droplet size and evaporation rate of water using the Comparative Kinetics Electrodynamic Balance for droplets generated from LB containing *E. coli* cells with a concentration of 6.6710^8 cells mL^{-1} into a gas phase at a RH of 33.2%. The initial droplet size is 27.8±0.08 μm.



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