H_2O_2 Kill Assays of Biofilm Bacteria

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[Abstract] Ubiquitous in nature and often surface associated, biofilms cause numerous chronic human infections. Biofilms are structured multicellular bacterial communities where cells are entrapped in a polymer matrix. Bacteria growing as biofilms are characterized by marked tolerance to many biocides, including oxidants such as hydrogen peroxide. Hydrogen peroxide is both produced by host phagocytic cells, and used as an antimicrobial compound. Understanding biofilm tolerance to hydrogen peroxide is therefore relevant to the persistence of *Pseudomonas aeruginosa* in human infections (such as chronic *Pseudomonas aeruginosa* infections in cystic fibrosis airways) as well as in environmental settings (such as water pipes).

This protocol was developed to determine the tolerance of *Pseudomonas aeruginosa* biofilms to hydrogen peroxide (H_2O_2) killing. The bacteria are grown as colony biofilms on polycarbonate membranes, as previously described in Walters *et al*. 2003. The protocol may be adapted for other bacterial, with appropriate changes in H_2O_2 concentrations, since different bacterial species may be more or less susceptible to H_2O_2 than *Pseudomonas aeruginosa*.

Materials and Reagents

1. 1x Phosphate buffered saline (PBS) solution, pH 7.4 (Sigma-Aldrich, catalog number: P4417-100TAB)
2. 30% w/w Hydrogen peroxide solution (undiluted, as sold commercially) (RICCA Chemical, catalog number: 3821.7-32)
3. 0.2% sodium thiosulfate solution (dissolved in ddH_2O) (Sigma-Aldrich, catalog number: S8503)
4. 0.2 µM Polycarbonate 25 mm membranes (General Electric Company, catalog number: K02BP02500)
5. *P.aeruginosa* strains in freezer stock
6. 25% Lennox broth (LB) medium (Becton Dickinson and Company, Difco™, catalog number: 240230) (see Recipes)
7. 25% LB agar plates (see Recipes)

Equipment

1. 6-well and 96-well plates
2. Standard petri plates
3. Spectrophotometer (cuvette) (Thermo Fisher Scientific, model: GENESYS 10S UV-Vis)
4. Spectrophotometer (96-well plate) (Bio-Rad, model: 680)
5. Cuvettes for OD_{600} reading
6. Shaking incubator at 37 °C, 250 rpm
7. Static incubator at 37 °C
8. Sterile glassware: 150 ml Erlenmeyer flasks, capped or foiled
9. 1.5 ml and 2 ml microcentrifuge tubes
10. Source of UV irradiation
11. Sterile wire-loops (sterilized with 70% ethanol and flame)
12. Stainless steel forceps (sterilized with 70% ethanol and flame)

Procedure

1. Day 0. Streak *P.aeruginosa* cells from the freezer stock onto a LB agar place and incubate statically overnight at 37 °C.
2. Day 1. Pick 4-5 single colonies from the *P.aeruginosa* agar plate with a sterile wired-loop and inoculate 15 ml liquid LB medium in a 150 ml Erlenmeyer flask. Grow liquid bacterial cultures overnight for 16-18 hours at 37 °C, with shaking at 250 rpm.
3. Day 2. Gently place polycarbonate membranes on agar surface of fresh sterile 25% LB agar plates and sterilize the membranes by placing them under UV irradiation for 1 hour. Handle membranes carefully with sterile forceps and use the membranes immediately after sterilization. Use eyes and skin UV protective equipment. Use at least 3 membranes per strain per condition for adequate biological replicates, and up to 6 membranes may be placed on each agar plate.
4. Measure the OD_{600} of the overnight bacterial culture and dilute the bacterial suspension in LB medium to a starting concentration of 10^8 cells/ml. Depending on the bacterial strain used, the OD_{600} to CFU ratio will differ and needs to be determined for each strain: for example, for the PAO1 wild type strain, 10^8 cells/ml = ~OD_{600} 0.1.
5. Spot 5 µl (5 x 10^5 cells) onto the sterile membranes and allow the liquid to be absorbed (10-20 minutes).
6. Incubate the colony biofilm on agar plates for 24 h at 37 °C.
7. Day 3. Using sterile forceps, gently lift the membranes off the agar surface and transfer them (cells side down) into 6-well plates filled with 2 ml of 25% LB liquid medium in each

well. Make sure the membranes are spread flat (*i.e.* not rolled up) and biofilm cells are remain on the membrane.

8. For the H_2O_2 treated biofilms, add 30 µl H_2O_2 (150 mM) to each well in pulses every 10 minutes for 30 minutes (for a final concentration of 450 mM H_2O_2 per challenge). The pulsing is done to mimic a continuous exposure of cells to H_2O_2. In between H_2O_2 pulses, incubate cells at 37 °C without shaking. Include untreated controls that are challenged with PBS. Each condition should be done at least in triplicates.
9. After H_2O_2 or PBS challenge, add 0.2% sodium thiosulfate to all samples to neutralize any remaining H_2O_2. Add even when samples are only challenges with PBS as a control.
10. To determine the viable cell count in H_2O_2 or PBS treated biofilms, collect biofilm cells by transferring the membranes and 2 ml of liquid from each well into 2 ml microcentrifuge tubes. Membranes are moved by gently lifting and rolling them using sterile forceps, with biofilm cells facing inward. The entire membrane should be submerged in liquid. Ensure to sterilize forceps between membrane transfers. Vortex biofilms in microcentrifuge tubes at maximal speed for at least 1 minute to detach and resuspend cells. Additional pipetting up and down and vortexing may also necessary to make sure there are no cell clumps visible in the bacterial suspension.
11. Aliquot 100 µl of bacterial suspension into 96-well plate, serially dilute cells 1:10, then plate 100 µl of each dilution on LB agar plates for CFU count. Incubate CFU count plates at 37 °C overnight.
12. Day 4. Count CFU on LB agar plates and calculate the viable CFU per biofilm based on the dilution factors applied.
13. Determine hydrogen peroxide killing by comparing the viable CFU count in the PBS treated and the H_2O_2 treated conditions.

Recipes

1. 25% LB medium
   5 g LB powder medium per L
   Dissolved in ddH_2O and autoclaved
2. 25% LB agar plates
   25% LB medium with 1.5% agar
   Dissolved in ddH_2O and autoclaved

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References

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