Voltammetric profiling of redox-active metabolites expressed by *Pseudomonas aeruginosa* for diagnostic purposes

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Supplemental information

Material and Methods

Bacteria and Growth Conditions

Wild-type *P. aeruginosa* strain PAO1 and the ΔpqsC + sPqsE mutant were used in this study. Each strain was grown overnight in LB medium at 20 °C under shaking conditions of 150 RPM, to an OD_{600} of 1.0. 1 ml of the culture was added to 9 ml of modified ABTG medium in a three-electrode electrochemical cell (EC). Briefly, the medium contained 15.1 mM $(NH_4)_2SO_4$, 33.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 0.05 mM NaCl, 1 mM $MgCl_2 \cdot 6H_2O$, 100 µM $CaCl_2$, 1 µM $FeCl_3$, and 28 mM glucose ($C_6H_{12}O_6$). The culture was then grown in the EC for 16-20 h at the specified potential at 30 °C under constant stirring of 500 RPM.

Electrochemical Setup and Analyses for viable *P. aeruginosa* cultures

Single chamber ECs with a working volume of 10 ml were operated in a three-electrode configuration on a VSP five channel potentiostat (Bio-Logic, France). ECs were assembled as previously described.[1] The counter electrode was a 0.1 mm Titanium wire (Sigma-Aldrich, Singapore) which was inserted into glass capillaries and soldered to a copper wire. The working electrode consisted of a 1 x 1 cm x 3.18 mm piece of Carbon felt (Alfa Aesar, USA) attached to the potentiostat via Titanium wire, a nylon screw and a nut (Small Part, USA). The resistance of each electrode assembly did not exceed 2.5 ohm. Prior to each experiment, the assembled ECs were sterilised at 121 °C for 20 min. The Ag/AgCl reference electrode (Alpha Analytical, Singapore) was connected to a 3 mm Vycor glass membrane (Alpha Analytical, Singapore) via a salt bridge consisting of 1 M KCl in 1.5% agar.

Chronoamperometry (CA), cyclic voltammetry (CV), and differential pulse voltammetry (DPV) were used to characterize the viable microbial cultures. CA was used to monitor cell growth by recording the current produced. Preliminary experiments on five-day old *P. aeruginosa* culture show negligible direct electron transfer following spent medium removal (data not shown). Thus, the current recorded was entirely attributed to mediated electron transfer via microbially-produced redox metabolites. Under these experimental conditions, estimations could be made regarding the growth phase the culture was in at any given time. CV and DPV were used to characterize the resultant metabolites produced during this growth. CV scan rate was 0.01 V/s; DPV parameters were: pulse height = 0.050 V; pulse width = 0.200 s; step

height = 0.002 V; step time = 0.400 s; scan rate = 0.005 V/s. CV and DPV were performed at the beginning of the experiment and after approximately 20 h, or as specified. In another series of experiments, growth cultures were poised at 0.1 V and 0.4 V. Control experiments were maintained at open circuit potential, i.e., native growth conditions. DPV was performed at 0, 5, 10, 15 and 20 h.

PQS standard addition and calibration

PAO1 after 20 h of growth at 0.100 V was centrifuged at 6000 RPM and filter-sterilized to obtain cell-free supernatant. This was then diluted x 5 in ABTG medium and added to a clean EC with a newly prepared CF electrode. DPV was conducted and the peak height at 0.23 V noted. Known concentrations of PQS standard were added (0.25-2 μM) with DPV performed after each addition. The increase in peak height was then plotted versus PQS concentration, allowing extrapolation to find the concentration in the starting supernatant.

H_2O_2 and MPII addition experiments in viable *P. aeruginosa* culture

PAO1 was grown overnight at 0.400 V and DPV was performed after 20 h. 10 μM microperoxidase MPII and H_2O_2 concentrations ranging from 1 mM to 5 mM were then added, with DPVs performed after each addition.

Electrochemical measurements without cells

Abiotic voltammetric experiments were conducted with a Metrohm Autolab PGSTAT 302N potentiostat using a three-electrode system in a glass cell (Metrohm). Working electrodes were 1 mm diameter planar glassy carbon disks (Cypress Systems) used in conjunction with a platinum counter electrode (Metrohm). Reference electrodes were either a silver wire miniature reference electrode (Cypress Systems) connected to the test solution via a salt bridge (containing 0.5 M Bu_4NPF_6 in DMSO) for non-aqueous solutions, or an Ag/AgCl (3 M KCl) reference electrode (Metrohm) for aqueous solutions. Prior to each scan, the solutions used for voltammetric analysis were de-oxygenated by purging with high purity argon gas, and the working electrodes were cleaned by polishing with alumina oxide (grain size 0.3 μm) slurry on a Buehler Ultra-pad polishing cloth, rinsing with ultrapure water, acetone, and then dried. All voltammetric experiments were conducted at 22±2 °C in a Faraday cage.

CV scans were conducted at 0.1 V/s. DPV parameters used were: step potential = 0.005 V; modulation amplitude = 0.025 V; modulation time = 0.05 s; interval time = 0.5 s; scan rate = 0.01 V/s.

CPE experiments in non-aqueous solution were performed in a two-compartment glass electrolysis cell divided by a sintered glass frit with a porosity number 5 (1.0–1.7 μm). Identically sized glassy carbon hollow cylinders were used as the working and auxiliary electrodes and symmetrically arranged with respect to each other with a silver wire reference electrode (isolated via a salt bridge containing 0.5 M Bu_4NPF_6 in DMSO) that was positioned to within 2 mm of the surface of the working electrode. The volumes of the solutions in both electrode compartments were approximately 25 mL each and

simultaneously de-oxygenated and stirred using bubbles of argon gas. All electrolysis experiments were conducted at 22±2 °C.

CPE was undertaken on PQS in DMSO (500 μM), PQS in HEPES buffer pH 7.4 (250 μM) and pyocyanin in HEPES buffer pH 7.4 (250 μM).

Pyocyanin oxidization experiments were undertaken on 5 mL solutions of either pyocyanin standard in HEPES buffer at pH 7.4 (250 μM), or filtrate from the *P. aeruginosa* PAO1 or ΔPQS +sPqsE strains cultivated in the bioelectrochemical system. Microperoxidase stock solution in DMSO (50 mM) was added to the 5 mL test solution to a final concentration of 5 μM. H_2O_2 concentration was then raised incrementally by adding aliquots of 100 mM stock solution in double distilled water.

Mass spectrometry analysis

Mass spectrometry analysis for the identification and quantification of PQS containing samples was performed in the positive mode on the Orbitrap Velos Pro (Thermo Scientific, USA) interfaced with high performance liquid chromatography (HPLC) pump (Accela 1250, Thermo Scientific, USA). For the identification and quantification 20 μL aliquots were injected by the Open Accela autosampler (Thermo Scientific, USA) into an Accela 1250 series HPLC and further separated on a Phenomenex Synergi 2.5 μ Fusion-RP 100 Å column (2.0 mm X 50 mm) by a 15 minute gradient program with a solvents flow-rate set at 300 μL/min. Solvent A was 50 μM EDTA (Sigma-Aldrich, Singapore) in water with 0.1% (v/v) formic acid (Fluka, Switzerland). Solvent B was 100% acetonitrile with 0.1% formic acid. A linear gradient from 2 % to 5 % of solvent B was applied during first 2 min. After that the concentration of solvent B was linearly ramped to 50% during 1 minute, increased linearly to 95% of solvent B during next step lasting 5.5 min and maintained at 95 % for 2 min. Finally, concentration of the solvent B was dropped down to the initial concentration of 2 % during 0.5 min and the column was allowed to re-equilibrate for additional 4 min. Each sample was measured once.

Quantitation was done using extracted ion chromatograms (XICs). Selected signal corresponding to ion of interest were extracted along the LC-MS run based on its exact m/z value (260.1650) with a deviation of 4 ppm. Extracted LC peak areas were integrated at a corresponding retention time (RT) by the XCalibur software (Thermo Scientific, USA). Concentrations of PQS were calculated based on the values of the XICs relative to the previously constructed calibration curve within its linearity range and adjusted to initial dilution.

Electron paramagnetic resonance (EPR)

Solution phase EPR spectra were collected on a Bruker ELEXSYS E500 EPR spectrometer operated in continuous wave X-band mode. DPPH samples (concentration = 1 mM) were prepared with and without PQS in equimolar ratio and transferred to a silica EPR flat cell with the following instrumental settings:

modulation amplitude = 0.1 G, sweep time = 30.72 seconds, time constant = 1.28 milliseconds and microwave power = 0.2 mW (corresponding to attenuation = 25 dB).

Results

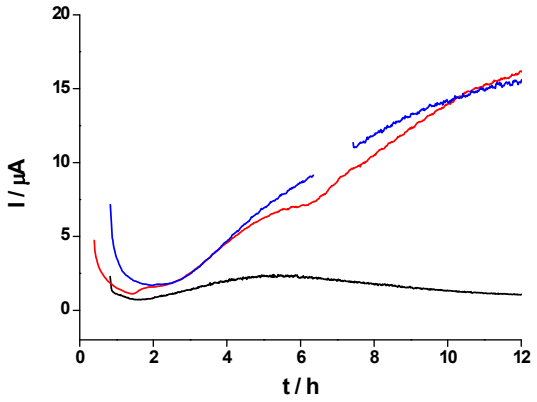

Fig. S1 Chronoamperometry of PAO1 grown at E = 0 V vs Ag/AgCl (black), 0.2 V (red) and 0.4 V (blue). pH 7.4, T = 30° C.

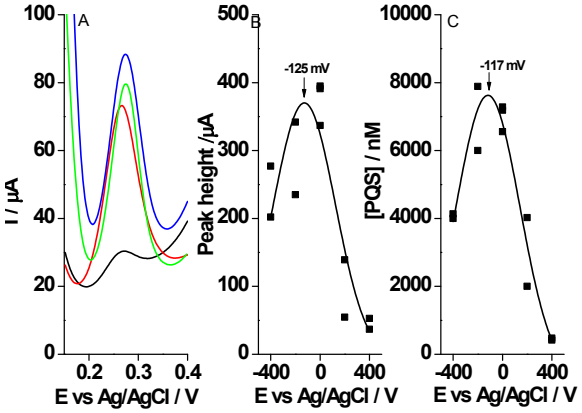

Fig. S2 (A) DPV of *P. aeruginosa* PAO1 across the potential of the putative PQS peak at t = 20 h, T = 30 °C, with working electrode poised at -0.2 V (green), 0.1 V (blue) and 0.4 V (black), and open circuit potential (red). (B) Maximum height of putative PQS peak from the DPV of *P. aeruginosa* PAO1 as a function of electrode poising potential. (C) PQS concentration of *P. aeruginosa* PAO1 at t = 20 h as a function of electrode poising potential as determined from LCMS/MS.

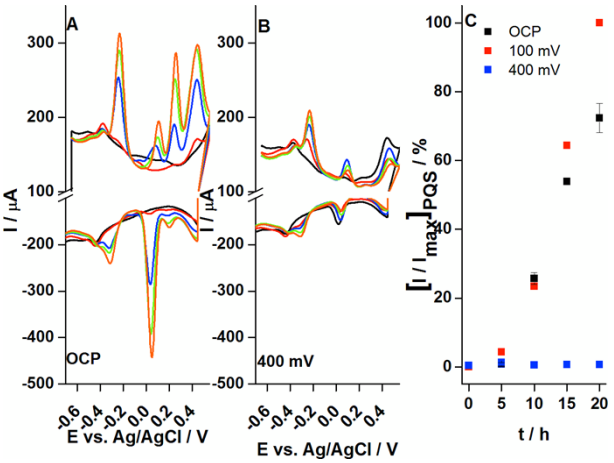

Fig. S3 DPV of *P. aeruginosa* PAO1 growth cultures on carbon felt electrodes at (A) open circuit potential (native conditions) and (B) 400 mV. (C) Height of PQS peak normalized to the maximum PQS peak height (at 100 mV), at 0 h (black), 5 h (red), 10 h (blue) and 15 h (green) and 20 h (orange). T = 30 °C.

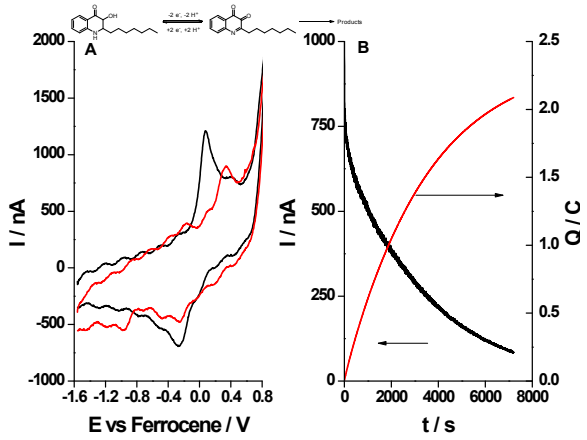

Fig. S4 (A) Cyclic voltammogram of 0.5 mM PQS in DMSO before and after controlled potential electrolysis showing disappearance of PQS peak at 0.08 V vs. ferrocene. (B) Controlled potential electrolysis of PQS in DMSO. T = 22 °C, E = 0.25 V vs. ferrocene, 0.1 V/s scan rate, 1 mm diameter planar glassy carbon electrode.

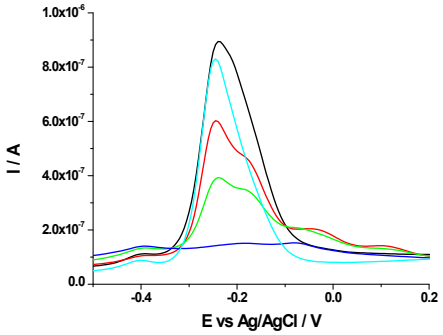

Fig. S5 DPV of pyocyanin standard in phosphate buffer solution (cyan) following microperoxidase (MPII) addition (black), MPII and H_2O_2 addition to 50 (red), 250 (green) and 1250 (blue) μM. pH 7.4, T = 22 °C, 0.1 V/s scan rate, 1-mm diameter planar glassy carbon electrode.

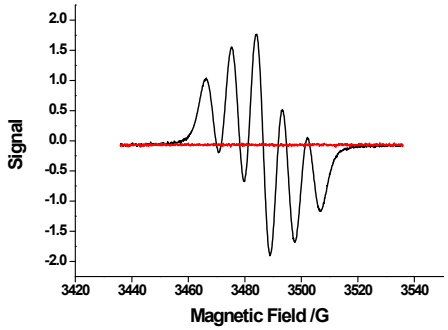

Fig. S6 Electroparamagnetic spectra generated from 1 μM DPPH radical in DMSO in the presence (black) and absence of PQS (1 μM) showing that PQS can reduce the DPPH radical and thus act as anti-oxidant. T = 22 °C.

References

1. E. Marsili, D. B. Baron, I. Shikhare, D. Coursolle, J. A. Gralnick, D. R. Bond, *Proc. Nat. Acad. Sci. USA*, 2008, **105**, 3968.