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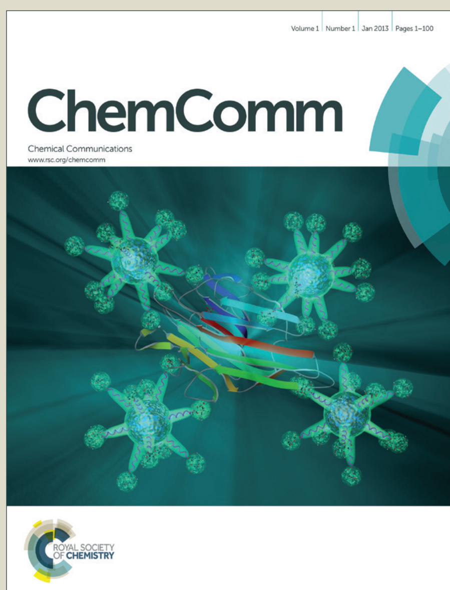

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Voltammetric profiling of redox-active metabolites expressed by *Pseudomonas aeruginosa* for diagnostic purposes

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In *Pseudomonas aeruginosa*, chemical deconvolution of the pyocyanin voltammetric signal allows its expression to be observed simultaneously with the quorum sensing molecule Pseudomonas quinolone signal (PQS). Such analysis revealed that PQS might protect pyocyanin from self-oxidation, but also exert a pro-oxidative effect on pyocyanin under oxidative conditions to produce additional redox metabolites.

Pseudomonas aeruginosa is a disinfectant- and antibiotic-resistant pathogen[1,2] that is common in natural and engineered habitats. The putative signalling molecule 2-heptyl-3-hydroxy-4-quinolone (i.e., Pseudomonas quinolone signal, or PQS)[3] and phenazines, such as pyocyanin,[4] contribute to the virulence of *P. aeruginosa*.[5] Pyocyanin can inactivate host proteases and is considered a direct determinant of *P. aeruginosa* virulence.[5,6] PQS, on the other hand, is thought to be only an indirect determinant of virulence through induction of *lasB* and *rhlI* expression, which encode the LasB elastase and N-butanoyl-L-homoserine lactone synthase (RhlI), respectively, thereby disrupting innate and adaptive immune systems.[3,7]

Pyocyanin and PQS are also redox-active, which has implications for the metabolism and extracellular defences of *P. aeruginosa*. Reversible cycling of pyocyanin between redox states at a potential (E) of approximately -200 mV vs. Ag/AgCl produces hydroxyl and superoxide radicals, which can damage epithelial cells.[8,9] Pyocyanin is also available to *P. aeruginosa* as a redox mediator, to maintain redox-balance under anaerobic conditions and to control the intracellular redox state.[10] The bisoxygenated aromatic structure of PQS allows it to act as an antioxidant which increases the tolerance of *P. aeruginosa* to oxidizing disinfectants.[11]

Regulation of pyocyanin and PQS expression by *P. aeruginosa* are interdependent. PQS and its effector gene (PqsE) control the biosynthesis of phenazines, while pyocyanin is itself the terminal-signalling molecule and contributes to upregulation of PQS and PqsE synthesis.[4,13] While it is clear that pyocyanin and PQS are linked at the level of genetic regulation, it is also possible that they interact via homogeneous electron transfer mechanisms. Reversible redox cycling of pyocyanin produces reactive oxygen species (ROS) that irreversibly oxidize part of the pyocyanin molecule.[14] As an anti-oxidant,[11] PQS may in fact therefore protect pyocyanin from this self-oxidation.

Voltammetric detection of pyocyanin has been reported, however it is unclear to what extent its voltammetric peak is obscured by the signal of other phenazines. Bukelman et al. (2009) described by electrochemistry the regulation of pyocyanin production by N-acyl homoserine lactones, another family of quorum sensing molecules.[12] Bellin et al. (2014) used a miniaturized sensing platform to spatially resolve the concentrations of phenazines in *P. aeruginosa* colonies.[15] Both assigned the dominant voltammetric signal, at -0.270 and -0.300 V vs. Ag/AgCl respectively, exclusively to pyocyanin, differently from previous findings that assigned the same peak only partially to pyocyanin.[16]

Electrochemical analysis of PQS has also been reported, in an abiotic system to nM concentrations using boron-doped thin-film diamond electrodes.[17] Simultaneous, real-time electrochemical detection of PQS and pyocyanin in growth cultures, however, has not been reported. Such detection, along with improved means to resolve the contribution of pyocyanin to the voltammetric signature of the phenazines, is required to further elucidate pyocyanin function and its chemical interplay with PQS. High-resolution detection of *P. aeruginosa* redox metabolites would help early diagnosis and treatment of sepsis and other life-threatening infections.[18]

P. aeruginosa PAO1 was grown in electrochemical cells poised at various electrode potentials and the current output was recorded (Fig. S1). The electrochemical profile of microbially-produced redox metabolites with time was determined through cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The DPV of *P. aeruginosa* PAO1 grown for 20 h at E = 0.1 V vs. Ag/AgCl shows three well-resolved redox peaks at -0.21±0.01, 0.11±0.01 and 0.25±0.01 V vs. Ag/AgCl (Fig. 1(A)). The peak centered at -0.21 V

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is consistent with that of phenazines detected on carbon electrodes in aqueous solution.[16,19] The peak centered at 0.25 V corresponds to PQS, as verified by standard addition method. The average PQS concentration in growth cultures calculated from a standard curve (Fig. 1(B) inset) was 3.32±2.04 μM (Fig. 1(B)).

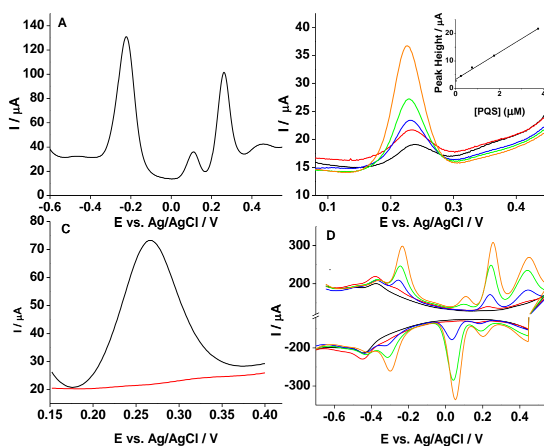

Fig. 1 (A) DPV at a scan rate of 0.005 V/s of *P. aeruginosa* PAO1. (B) PQS determination in the supernatant of *P. aeruginosa* PAO1. (C) DPV of *P. aeruginosa* PAO1 (black trace) and its ΔPQS mutant (red trace). (D) DPV at a scan rate of 0.005 V/s of *P. aeruginosa* PAO1 at 0 h (black), 5 h (red), 10 h (blue) and 15 h (green) and 20 h (orange) showing the increase of PQS concentration with time. *P. aeruginosa* cultures were grown on carbon felt electrodes for 20 h at E = 0.1 V vs. Ag/AgCl and 30 °C.

The DPV of the *P. aeruginosa* PAO1 grown at 0.1 V vs. Ag/AgCl was recorded at t = 0, 5, 10, 15 and 20 h (Fig. 1(D)). Following a brief lag period, the concentration of both phenazine and PQS increased with time. The DPV of the ΔPQSc mutant lacking PQS expression did not display the peak at 0.25 V after 20 h (Fig. 1(C)). Thus, PQS expression in *P. aeruginosa* growth cultures can be determined electrochemically on carbon felt electrodes. The peak centered at 0.11 V corresponds to a biomass-adsorbed species, as it does not appear in the cell-free filtrate (data not shown). Therefore, this peak was not assigned to any of the *P. aeruginosa* metabolites tested in this work.

Although PQS is known to be irreversibly oxidized at E > 0.8 V on a diamond electrode,[17] the effect of mild oxidative potentials on PQS concentration is not known. Here, *P. aeruginosa* was grown on electrodes poised at potentials between -0.2 V and 0.4 V vs. Ag/AgCl. The PQS concentration was measured based on peak height relative to a standard curve and it decreased as potential increased from 0.0 to 0.4 V vs Ag/AgCl. The maximum PQS concentration after 20 h of growth was observed at -0.1 to 0 V vs. Ag/AgCl (Fig. S2(A)) with good correlation between peak amplitude and PQS concentration as determined by LCMS/MS (Fig. S2(B) and S2(C)), further confirming that PQS can be reliably detected electrochemically. PQS concentration was determined at five time points (0, 5, 10, 15 and 20 h) at open circuit potential (OCP, native conditions), and 0.4 V (Fig. S3). The time-dependent accumulation of phenazine at 0.4 V vs. Ag/AgCl was not accompanied by an induction of PQS expression (Fig. S3B). Under OCP conditions (Fig. S3A), both phenazine and PQS peak increased with time. The comparison of normalised peak heights for PQS showed that PQS expression and accumulation was slightly higher at 0.1 V than under OCP conditions (Fig. S3C). It is not clear whether the change in PQS concentration with electrode potential is due to molecular regulation, chemical interaction between PQS and other redox metabolites such as pyocyanin, electrochemical oxidation of PQS or a combination of these effects. The expression of PQS and phenazine with time at OCP is consistent with a low potential bias, as previously observed. [12]

While it is important to be able to detect the bulk concentration of PQS in an aqueous system, local concentrations of PQS will likely be much higher as PQS can be transported through the extracellular biofilm milieu within membrane vesicles.[20] Studying the redox chemistry of PQS in abiotic experiments employing organic solvents was thus employed in this study to better understand local interactions between PQS and pyocyanin.

A CV of 0.5 mM PQS in 25 mL DMSO was recorded prior to and after the oxidative Controlled Potential Electrolysis (CPE) (Fig. S4(A)). A potential, approximately 0.15 V more positive than the peak oxidation potential value of PQS obtained by CV, was applied for 2 h until 88 % PQS oxidation was achieved (Fig. S4(B)). The oxidation and reduction peaks were detected at 0.08 and -0.26 V respectively vs. ferrocene. After CPE, the oxidation peak at 0.08 V was greatly diminished (or almost completely absent) and other minor redox peaks appeared. Since the PQS redox reaction is poorly reversible over the long period of CPE, it is suggested that the reduction in PQS detection at poised electrode potentials > 0.1 V vs. Ag/AgCl was due to irreversible oxidation of PQS. PQS oxidation involves 2 electrons, as calculated from CPE data using the Faraday equation, which is consistent with the reaction mechanism previously postulated for PQS oxidation[11] (Fig. S4(A) inset).

Reszka et al. (2004) demonstrated the irreversible oxidation of pyocyanin by hydrogen peroxide (H_2O_2) in the presence of several peroxidases.[14] This oxidation targeted the phenolate group and is specific for pyocyanin. We therefore postulated that irreversible oxidation by H_2O_2 could allow the contribution of the pyocyanin to the voltammetric profile of *P. aeruginosa* to be decoupled from the other phenazines.

The abiotic, irreversible oxidation of pyocyanin by H_2O_2 addition is described in Fig. S5. A H_2O_2 concentration-dependent quenching of the pyocyanin signal was observed in the DPV of pyocyanin in PBS buffer only in the presence of 5 μM microperoxidase (MPII). Quenching results in the formation of oxidation byproducts that have peaks at 0.1 and -0.05 V Ag/AgCl. These byproducts, however, appear to have low stability and their signals are short-lived as indicated by their complete disappearance following the increase in H_2O_2 concentration to 1.25 mM.

In the presence of 10 μM MPII, H_2O_2 was added at concentrations of 50, 250 and 1250 μM to sterile filtrated supernatants of *P. aeruginosa* grown at 0.1 V vs. Ag/AgCl and harvested at the onset of stationary phase (Fig. 2(A)). H_2O_2 addition up to 250 μM reduces the signal at -0.21 V, but no further reduction in the peak was observed at higher H_2O_2 concentrations. These data suggest that pyocyanin contributes approximately 50% to the area of the phenazine peak in the DPV and demonstrate that this method can be used to remove interference from other phenazines to estimate the amount of pyocyanin present.

It has been previously shown that over-expressing PqsE in a PQS-negative *P. aeruginosa* mutant (i.e. ΔpqsC + sPqsE) results in the over-synthesis of phenazines and increase in current production.[21] H_2O_2/MPII addition experiments were therefore repeated using cell-

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free filtrate from the ΔpqsC + sPqsE *P. aeruginosa* mutant, to determine the extent to which pyocyanin contributes to the increase in phenazine production induced by the PqsE. While a ten-fold increase in intensity of the phenazine peak for the wild-type was observed (Fig. 2(B)), only a small reduction in peak height was observed following the addition of 5 mM H_2O_2. When pure pyocyanin standard was added to the filtrate, the electrochemical signal was at a higher potential (-0.20 compared to -0.24 V vs. Ag/AgCl for the dominant phenazine peak) and was readily oxidized by MPII/ H_2O_2 addition. In contrast, there was no further reduction in the intensity of the putative phenazine peak. Thus, while a functional PqsE increases the pyocyanin concentration,[21] other redox-active molecule/s with similar electrochemical potential appear to be significantly upregulated as a consequence of the over-expression of PqsE.

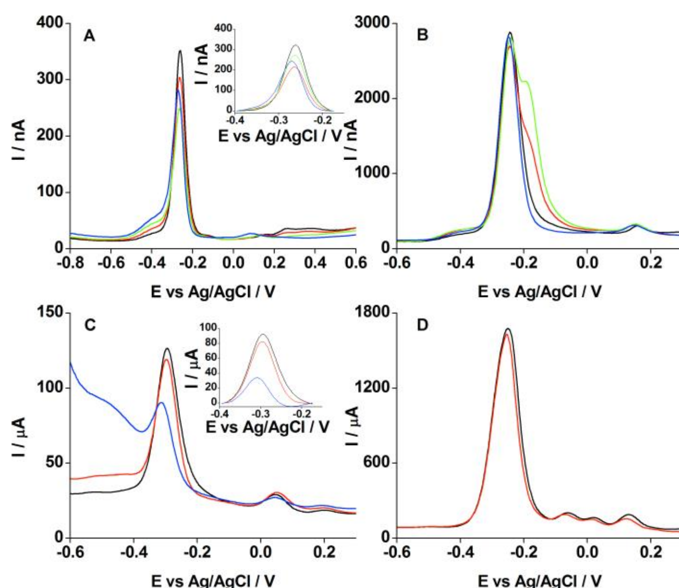

Fig. 2 (A) DPV of *P. aeruginosa* PAO1 filtrate (black) following MPII and H_2O_2 addition to 50 (red), 250 (green) and 1250 (blue) µM with baseline correction for the phenazine peak (inset). (B) DPV of *P. aeruginosa* PAO1 ΔpqsC + sPqsE filtrate (black) following MPII and H_2O_2 addition to 2500 µM (blue), pyocyanin standard addition (200 µM), MPII and H_2O_2 addition to 2500 µM (green) pyocyanin standard addition (200 µM), MPII and H_2O_2 addition to 5000 µM (red). (C) DPV of *P. aeruginosa* PAO1 growth culture (black) following MPII and H_2O_2 addition to 1 (red) and 10 (blue) mM with baseline correction for the phenazine peak (inset). (D) DPV of *P. aeruginosa* PAO1 ΔpqsC + sPqsE growth culture (black) following MPII and H_2O_2 addition to 10 mM (red). (A, B) t = 20 h, pH 7.4, T = 22 °C, 0.1 V/s scan rate, 1-mm diameter planar glassy carbon electrode. (C, D) t = 20 h, pH 7.4, T = 30 °C, E = 0.1 V vs. Ag/AgCl, 0.005 V/s scan rate, carbon felt electrode.

LCMS/MS analysis was undertaken on the ΔpqsC + sPqsE mutant (grown at 0.1 V vs. Ag/AgCl) to quantify the three remaining phenazines expressed by *P. aeruginosa*. A two-fold increase in only phenazine-1-carboxylic acid (PCA) was observed, with no increase detected for phenazine-1-carboxamide. 5-MCA is not considered sufficiently stable for chemical analysis by LCMS/MS and was not quantified here.[15] The three-fold increase in current previously observed[21] may therefore be explained by upregulation in the expression of other metabolites. PqsE is known to regulate lectin synthesis. Lectin from *P. aeruginosa* as well as quorum sensing molecules known to play a role in phenazine synthesis, N-3-oxo-dodecanoyl-L-homoserine lactone and N-butanoyl-l-homoserine lactone, were screened for electrochemical responses, but none of these molecules displayed a voltammetric response. Nonetheless, deconvoluting the pyocyanin peak from the other phenazines in a real-time bioelectrochemical cell allows us to confirm pyocyanin as only a small contributor to the phenazine peak in the Δ*pqsC* + sPqsE strain, and therefore a minor component of the increase in phenazines induced by the PqsE effector relative to the wild-type strain.

Pyocyanin peak-deconvolution by MPII/H_2O_2 was also undertaken in the presence of cells of the wild-type PAO1 and the Δ*pqsC* + sPqsE mutant. As indicated by Fig. 2(C) and 2(D), a large reduction in the phenazine peak was observed in the wild-type and no reduction in the peak observed in the Δ*pqsC* + sPqsE, consistent with what was observed in the filtrate.

It has been shown that PQS can act as an anti-oxidant, due to its bisoxygenated aromatic structure that readily donates electrons to form an oxidized ortho-quinoid. To verify this, electron paramagnetic resonance (EPR) spectra were recorded for the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with and without the addition of equimolar amounts of PQS (Fig. S6). The data show that PQS quenches the DPPH radical, providing further evidence of its ability to act as an anti-oxidant. Pyocyanin is susceptible to self-oxidation by hydroxyl radicals generated during redox cycling. Hence, it is possible that PQS mitigates this self-oxidation. The loss of an anti-oxidant like PQS could therefore leave pyocyanin more susceptible to irreversible oxidation.

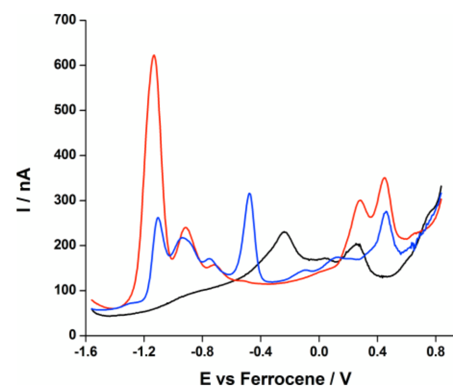

Fig. 3 DPV of oxidized PQS 0.5 mM (black) after controlled potential electrolysis, pyocyanin 0.5 mM (red) and pyocyanin 0.5 mM mixed with oxidized PQS 0.5 mM (blue), showing 80% depletion of pyocyanin oxidation peak and oxidation of pyocyanin by oxidized PQS. DMSO, T = 22 °C, scan rate 0.1 V/s, 1-mm diameter planar glassy carbon electrode.

Another possible consequence of the ability of PQS to act as an anti-oxidant is that it could have a pro-oxidative effect. Irreversibly oxidized PQS could in turn irreversibly oxidize pyocyanin. Such a model is supported by the DPV measured following the mixture of equimolar amounts of oxidized PQS with pyocyanin (Fig. 3) where an 80% reduction in the amplitude of the pyocyanin peak was observed concomitant with the appearance of a new voltammetric peak at -0.5 V, which is consistent with the production of an oxidative byproduct. Since it was shown above that PQS oxidation was irreversible, this new peak is indicative of the oxidation of pyocyanin.

As described here, poising the electrode at potentials greater than 0.25 V vs. Ag/AgCl leads to irreversible PQS oxidization. In contrast, pyocyanin is not irreversibly oxidized at potentials ≤0.4 V. The net effect of irreversible PQS oxidation on pyocyanin expression by *P. aeruginosa* can therefore be observed by growing cultures at E >0.25V. Pyocyanin concentrations were compared

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using electrochemical peak deconvolution in cultures grown at E = 0.1 V and 0.4 V vs. Ag/AgCl and OCP. A corresponding decrease in phenazine signal intensity was observed at 0.4 V relative to OCP and 0.1 V vs. Ag/AgCl (Fig. S3). A reduction of 5 % in the phenazine peak following MPII/H_2O_2 addition was observed for cultures grown at 0.4 V compared to approximately 50 % for those grown at -0.2 V. Pyocyanin therefore constitutes a very small component of the total phenazines when *P. aeruginosa* is grown at E > 0.25 V, accounting for the reduction in intensity of the phenazine peak compared to growth at -0.2 V.

The concentration of pyocyanin detected in growing cultures is therefore dependent on the applied potential and increases as the working electrode potential decreases, i.e. as the energetic payoff for the cell decreases. This observation could be explained in terms of upregulation of the pyocyanin at lower electrode potential, to increase extracellular anodic respiration in response to this limitation in terminal electron acceptor. However, a second, more likely, explanation is that it results from partial or complete PQS oxidization at high electrode potential (E > 0.2 V); due to the combined effects of pyocyanin synthesis being downregulated, depletion of PQS leaving pyocyanin more susceptible to self-oxidation and, perhaps more likely, oxygen radicals produced in the PQS oxidation in turn oxidizing pyocyanin. Experiments with poised growing cultures allow controlling the energetic payoff and possibly the growth rate of the cells and help unveiling the interplay between biological and redox regulation in the production of extracellular metabolites.

Interactions between PQS and pyocyanin by homogeneous electron transfer mechanisms have been demonstrated here and should be considered in addition to the regulatory interactions already reported to explain changes in PQS and pyocyanin concentrations during growth. While the biological importance of the electrochemical interactions remains to be fully elucidated, such interactions are implicitly significant by the fact that the regulatory mechanisms of PQS and pyocyanin are strongly interdependent. Regardless of whether or not these interactions have been evolutionarily selected and what their consequences are, the data presented here clearly show that PQS and pyocyanin are biochemically connected through redox cycling and oxidative stress responses.

Bioelectrochemical detection of redox metabolites in microbial growth cultures is sensitive and requires little or no sample preparation. The means to characterize a greater range of *P. aeruginosa* metabolites and their chemical and biological interactions broadens the possibility for the application of electrochemical sensors in patient diagnostics. Additionally, it increases our understanding of the mechanisms of *P. aeruginosa* virulence. We have provided three examples of the diagnostic possibilities for electrochemical profiling with regards to describing aspects of *P. aeruginosa* metabolism. Further research in this area should expand the range of microbial metabolites that can be detected through electrochemical methods, thus leading to rapid characterization of *P. aeruginosa* infections in healthcare settings.

Notes and references

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