Cyclic voltammetric, fluorescence and biological analysis of purified aeruginosin A, a secreted red pigment of Pseudomonas aeruginosa PAO1

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The opportunistic pathogen Pseudomonas aeruginosa produces multiple pigments during in vitro culture and in vivo during colonization of burn wounds and in the airways of cystic fibrosis (CF) patients. One pigment is a deep ‘merlot’-coloured compound known as aeruginosin A (AA). However, the red pigment(s) of P. aeruginosa are often collectively called pyorubrin, of which there is no known chemical composition. Here, we purified and confirmed by MS and assessed the physicochemical properties of AA (2-amino-6-carboxy-10-methylphenazinium betaine) by first focusing on its ability to redox-cycle using cyclic voltammetry and its spectroscopic (as well as fluorescent) properties, experiments that were conducted at physiological pH. AA exhibited reversible electrochemistry at a glassy carbon electrode within a potential range of ~500 to ~200 mV. Electrochemical anodic and cathodic peak currents were observed at ~327 and ~360 mV, respectively, with a low formal reduction potential of ~343.5 mV versus Ag/AgCl. AA absorbed at 516 nm and fluoresced at 606 nm. Results from the spectro-electrochemistry of pyorubrin revealed that its strongest fluorescence was in its parent or oxidized form. Production of AA by P. aeruginosa was found to be controlled by the rhl component of the intercellular signalling system known as quorum sensing and was produced maximally during the stationary growth phase. However, unlike its downstream blue redox-active toxin, pyocyanin, AA had no adverse effects on methicillin-resistant Staphylococcus aureus USA300, Escherichia coli DH5α or human keratinocytes. We close with some thoughts on the potential commercial use(s) of AA.

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen that is capable of causing a number of highly problematic infections of burns, wounds, catheters, valves, tubing and stents. It is particularly hazardous for patients whose immune systems have been compromised such as those who are HIV positive or undergoing cancer chemotherapy or those with chronic alcoholism. However, it is during airway infection of cystic fibrosis (CF) patients where the organism has gained its greatest notoriety.

P. aeruginosa produces multiple pigments during routine culture in vitro and often during in vivo infection of CF and burn patients. Many of these pigments are also known to play a significant role in the virulence (disease-causing) properties of the organism. The most notable pigment, pyocyanin (PYO), is a blue, redox-active pigment that is most prominently produced when organisms are starved of phosphate (Hassan & Fridovich, 1980; Hassett et al., 1992), necessary for the biosynthesis of cellular ATP. PYO kills other bacteria in a variety of competitive niches especially when in complex, highly organized communities known as biofilms and also damages human airway epithelial cells, in part by inactivation of vacuolar ATPase activity (Ran et al., 2003). Pyochelin and pyoverdin are colourless and green fluorescent siderophores, respectively, that are appropriately produced when bacteria are starved of iron (Cox, 1979; Meyer et al., 1992). These compounds help P. aeruginosa obtain iron from the host during infection, and
strains lacking the ability to produce these compounds often have significantly impaired virulence properties (Meyer et al., 1996; Takase et al., 2000). Pyomelanin is a brown to black compound resulting typically from mutations within genes involved in the tyrosine degradation pathway (Jensen & Pierson, 1975). For example, when P. aeruginosa accumulates homogentisate due to mutations within hmgA encoding homogentisate-1,2-dioxygenase (HmgA), under slightly alkaline conditions homogentisate accepts an electron and ultimately polymerizes into a large black phenyl-propanolike moiety termed pyomelanin, via a free radical chain reaction. Pyomelanin is known to have antioxidant properties, similar to melanins of eukaryotic origin such as humans and in the fungi Cryptococcus neoformans and Histoplasma capsulatum (van Duin et al., 2002).

Red-pigmented P. aeruginosa strains have also been isolated from the environment and infected patients (Brown & Lowbury, 1965; Phillips, 1969). Aeruginosin A (AA) and B (5-methyl-7-aminocarboxymethylphenazinium betaine and 5-methyl-7-amino-1-carboxy-3-sulfo-methylphenazinium betaine, respectively) are two red pigments produced by P. aeruginosa after prolonged aerobic incubation (Thomassen et al., 1979). Despite being a phanenzine, AA and aeruginosin B are highly water-soluble and not extractable in chloroform, yet can easily bind to activated charcoal and alumina, indicative of the molecule being highly hydrophilic (Herbert & Holliman, 1969; Holliman, 1969). Besides red pigments AA and aeruginosin B, Mavrodi et al. (2001) found that a phzS mutant of P. aeruginosa, devoid of pyocyanin hydroxylase, produced large amounts of an unknown dark red, water-soluble pigment, which was hypothesized not to be AA and aeruginosin B, but rather a coloured intermediate(s) that they postulated to be 5-methylphenazinium-1-carboxyl acid betaine (5MPCA). In addition, a phanenzine 5MPCA-derived antifungal red pigment, different from AA and aeruginosin B, was reported to develop and accumulate within fungal cells in either P. aeruginosa–Candida albicans co-cultures grown on solid medium or in Candida albicans grown on agar containing phanenzine methosulphate (PMS), a 5MPCA analogue (Gibson et al., 2009; Morales et al., 2010).

Despite numerous studies on the multiple pigments of P. aeruginosa, especially the well-studied PYO (due primarily to its toxic properties), very little is known of the biological function of AA. In this study, we first assessed the redox properties of P. aeruginosa AA and demonstrated that AA has significant redox-active and fluorescent properties. However, unlike the blue redox-active antibiotic PYO, AA lacks antimicrobial or cytotoxic properties under physiological conditions. Thus, we offer some potential uses for AA for human use, potentially in the very lucrative cosmetic industry.

**METHODS**

**Bacterial strains and planktonic growth conditions.** All bacteria used in this study are listed in Table 1. Organisms were grown at 37 °C in either Luria–Bertani broth (L-broth) or a low-phosphate succinate medium (LPSM), a formulation that is optimized for overproduction of AA at 37 °C with shaking at 300 r.p.m. (Cox, 1986; Hassett et al., 1992). Culture volumes were typically 1/10 of the total Erlenmeyer flask volume to ensure maximum aeration. Media were solidified with 1.5 % Bacto agar. Frozen bacterial stocks were stored at −80 °C in a 1:1 mixture of 30 % sterile glycerol and stationary-phase bacteria.

**Purification of AA from P. aeruginosa.** AA was purified from culture supernatants from a phzS mutant of P. aeruginosa PAO1 (Mavrodi et al., 2001) as described by Holliman (1969), who used a red-pigmented strain. Simply, 100 ml LPSM was inoculated with the P. aeruginosa phzS mutant and allowed to grow for 48 h at 37 °C. Bacteria were clarified by centrifugation at 10 000 g for 15 min at 4 °C. The supernatant was extracted twice with the same volume of chloroform. Celite 535 (2 g) was added to the deep red aqueous supernatant and incubated for 30 min at room temperature, and the mixture was centrifuged at 5000 g for 5 min. The pH of the supernatant was adjusted to 4.5 with HCl. Activated charcoal (2 g) was added to the red supernatant and vortexed at 4 °C for 1 h. The charcoal-bound pigmentation was then collected by centrifugation, mixed thoroughly with 2 g Celite 535 in 50 ml double distilled H2O (ddH2O), and carefully layered onto a 50 ml plastic cylindrical column. The packed Celite 535 column was washed extensively with ddH2O followed by removal of impurities with 50 % ethanol. The AA fractions were eluted with 5 % pyridine in a fume hood, and the eluate was removed from the pyridine by extraction with ether and then air-dried.

**TLC analysis.** The dried powder from the above procedure was solubilized in 1 ml ddH2O. AA (20 μl) was spotted onto an analytical TLC plate (Silica Gel 60 A, 20 × 20 cm, Partisil K6; Whatman), which was then fully developed with 70 % methanol in a developing chamber with a glass lid to maintain moisture (Cole Parmer Instrument Co.). The developed plate was then air-dried and photographed. Three pigmented bands were observed without any staining required (Fig. 1b). To further analyse these pigments, a larger preparative TLC plate from 2 litres of LPSM-grown culture was allowed to separate longer and developed in the same fashion and the pigments air-dried. Each pigment band was carefully recovered from the TLC plates with a brand new steel single-edged razor blade and dissolved in an appropriate solvent (either water or 15 % ethanol). The silica particles were removed by centrifugation and the clear supernatants were subjected to further MS analysis.

**MS analysis.** All experiments were performed on a Thermo Scientific LTQ-FT, a hybrid instrument consisting of a linear ion trap and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. The sample was diluted with 1 part water and 2 parts ethanol and directly infused into the LTQ-FT using the standard electrospray ionization source for the instrument with a spray voltage of 5 kV and a capillary temperature of 275 °C. Mass accuracy errors for AA were below 100 p.p.b. MSn spectra were obtained in the linear ion trap by collisions with helium at normalized collision energy of 35. MSn mass accuracy errors measured in the FT-ICR mass spectrometer were below 1000 p.p.b.

**Electrochemical measurements.** Bioanalytical Systems BAS-100B, BASI Epsilon BC and rotating disc electrode RDE-2 workstations were used for all cyclic voltammetric experiments. A glassy carbon rotating disc electrode (3 mm diameter) from Bioanalytical Systems was used as the working electrode. An Ag/AgCl wire (0.5 mm diameter) was used as the reference electrode and a Pt wire (0.5 mm diameter) was used as the auxiliary electrode. For all electrochemical experiments, purified AA was buffered in 0.05 M phosphate and 0.1 M NaCl (pH 7.0). An Accumet Excel XI Dual Channel pH/ion/conductivity meter.
meter (Fisher Scientific) was used to measure the buffer pH. A 30 μl drop of the analyte was placed on a microscope slide, which had been coated with surfacil to create a hydrophobic surface and the rotating disc electrode was lowered to squeeze the drop between the slide and the electrode. Sweep rates from 5 to 125 mV s⁻¹ were used for cyclic voltammetry.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Reference or source of strain</th>
</tr>
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<tbody>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type strain, prototroph</td>
<td>Holloway (1955)</td>
</tr>
<tr>
<td>lasI</td>
<td>lasI::Gm</td>
<td>Hassett et al. (1999)</td>
</tr>
<tr>
<td>rhlI</td>
<td>rhlI::Gm</td>
<td>Hassett et al. (1999)</td>
</tr>
<tr>
<td>lasI rhlI</td>
<td>lasI::Gm, rhlI::Tc</td>
<td>Hassett et al. (1999)</td>
</tr>
<tr>
<td>phzS</td>
<td>phzS::Gm</td>
<td>Mavrodi et al. (2001)</td>
</tr>
<tr>
<td>E. coli DH5-α</td>
<td>F⁻ 4800lacZΔM15 endA1 recA1 hsdR17(rK mK⁺) supE44 thi-1 gyrA96 Δ(lacZYA-argF)U169</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S. aureus USA300</td>
<td>A renowned MRSA strain</td>
<td>ATCC</td>
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Fig. 1. (a) Enzymic cascade of phenazine biosynthesis in *P. aeruginosa* (adapted, with permission, from Mavrodi et al., 2001), specifically relating to the bisection using ammonia catalysis of AA production from 5MPCA. (b) Thin layer chromatogram showing purified AA. (c) Mass spectrogram of purified AA. (d) Chemical structure of purified AA (Holliman, 1969).
Spectroscopic measurements. A Varian Cary 50 Bio UV-Visible Spectrophotometer was used to obtain UV–Vis absorption spectra. A Varian Cary Eclipse fluorescence spectrophotometer was used to obtain the emission spectra in a cuvette. Emission spectra on a drop were obtained using a 6-around-1 fibre optic bundle laser (441.6 nm HeCd model 1K4153R-C; Kimmon Electric Co.), light control modules (shutter, attenuator and focusing optics), a monochromator (0.3 m focal length, triple grating turret) and photon-counting phototube (Acton Research Corp.), and a computer with data acquisition and analysis software (NCL and Spectra-Sense software; Acton).

Assessment of the role of quorum sensing (QS) in AA production. P. aeruginosa last, rhl and last rhl mutants were constructed as described by Hassett et al. (1999). Bacteria were grown aerobically in LPSM in the absence or presence of P. aeruginosa homoserine lactone signalling molecules (Pseudomonas autoinducer) PAI-1 or PAI-2 or both autoinducers for up to 48 h at 37°C. The final concentrations in solution of PAI-1 and PAI-2 were 2 and 3.8 μM, respectively, amounts known to be produced in stationary-phase cultures (Hassett et al., 1999). Bacteria were clarified by centrifugation, and the culture supernatants were diluted 1:1 with chloroform and vortexed for 30 s. PYO is easily extractable in the organic phase while AA remained in the aqueous phase. AA and PYO were plotted as AA (OD520), PYO (OD690) and turbidity (OD750).

Effect of AA on bacteria. E. coli DH5α and S. aureus USA300 (a methicillin-resistant strain; Centers for Disease Control and Prevention, 2003) were grown in L-broth under aerobic conditions until stationary phase. The suspensions were diluted 1:100 in 3 ml molten L-agar containing 0.8% low-melting-point agarose at 45°C and the suspension poured onto L-agar plates. Once solidified, the plates were inverted and incubated at 37°C for 24 h. The zone of inhibition in triplicate were then determined based upon dilution of nanomolar quantities of PYO and AA.

Effect of AA on human keratinocyte viability. Cell number (i.e. viability) was determined by direct counting. For this, keratinocytes were seeded at 5.5 x 10⁴ cells per 12.5 cm² flask. Cells were allowed to attach and grow for 48 h before treatment. Cells were then treated daily with fresh growth media containing test compounds or vehicle control (1% ethanol) for 5 days. Compounds were tested at a range of concentrations to determine the maximum dose that did not affect viability of human keratinocytes. On the sixth day, cells were detached with 1 x trypsin/EDTA and counted with a Coulter Counter. To determine the fraction of cells surviving the treatment in a specified concentration of three fractions (red, brown, pink), the viable cell number after each treatment was normalized using the average of the viable cell number in a control group.

RESULTS

Purification of AA from P. aeruginosa

The original proposed mechanism for the biosynthesis of P. aeruginosa blue toxin PYO is shown in Fig. 1(a) (Mavrodi et al., 2001). It was necessary to include this figure with permission from the authors and the Journal of Bacteriology to show the various steps that could lead to the production of the red pigment, AA. Holliman (1969) more than 40 years ago elucidated the structure of AA (2-amino-6-carboxy-10-methylphenazinium betaine). We have also purified and confirmed its structure, and our study revealed some unique redox, fluorescent and non-toxic (bacteria and human cells) properties of AA that were previously unrecognized. For optimal production of AA, we elected to use an isogenic phzS mutant of wild-type P. aeruginosa strain PAO1 (Mavrodi et al., 2001). The PhzS protein encodes a pyocyanin hydroxylase that catalyses the conversion of 5-methyl-phenazine-1-carboxylic acid to pyocyanin as part of the phenazine biosynthesis cascade (Fig. 1a). When grown in LPSM (see Methods), this strain is forced genetically to overproduce the water-soluble red fraction containing AA. If wild-type bacteria were used, there would be a chloroform-soluble ‘blue’ fraction containing PYO. However, the ‘red’ fraction also contained other pigments (e.g. brown, pink, red) as well (also see Fig. 6 below). For example, note in Fig. 1(b) that there are three bands that are separated after TLC analysis (Fig. 1b), the slowest migrating (upward bands migrate more rapidly) species (pink colour) being defined by MS as AA. An accurate mass spectrum obtained in the FT-ICR portion of the LTQ-FT is shown in Fig. 1(c). AA (C14H23N4O5) is observed at m/z 254.09241 (theoretical=254.09240; error=26 p.p.b.), and its sodium salt, C15H25N4O5Na+ (Fig. 1d, without Na+, 2-amino-6-carboxy-10-methylphenazinium betaine), is observed at m/z 276.07434 (theoretical=276.07435; error=−29 p.p.b.). These data confirm the elemental composition for these two components and are the only possibilities for C9–39H0–60N0–10O0–15N0–40–1+ with an error of 2 p.p.m. or less. Decarboxylation products were also observed. These compounds may be present in the sample or formed when introducing the ions into the instrument. The latter possibility is supported by the fact that the major product ion observed in collision-induced dissociation is decarboxylation of the parent compound. The middle red band was identified by MS as phenazine-1-carboxylic acid (PCA), which typically displays a very pale yellow colour when oxidized, yet is bright yellow when reduced in aqueous solution. It is uncertain whether the reddish colour of PCA formed on TLC was a solvent effect due to methanol in the developing system. Yet AA and PCA were observed in the crude, column-purified and TLC-purified fractions (data not shown). Why we are observing different colours is unknown. It may be that while PCA is the dominant species in that region of the TLC plate, a less abundant but more intensely coloured species is also present; red would also easily mask the yellow. It may also be a function of pH [similar compounds such as ‘neutral red’ (toluylene red) – pH <6.8 is red, pH >8.0 is yellow], solvent, impurities such as metal ions or other factors. The decarboxylation of AA shown in Fig. 1(c) may be an instrumentation artefact, i.e. collision-induced dissociation upon acceleration in a relatively high-pressure region prior to introduction to the source. This possibility is further supported by the favourable decarboxylation observed in the MS² of AA. Unfortunately, we could not define the structure of the upper brown band despite extensive attempts to do so.

Cyclic voltammetric analysis of AA

To study the electrochemistry of AA, cyclic voltammetric experiments were performed. Cyclic voltammetry is usually
the first electroanalytical technique used to search and characterize redox couples in an uncharacterized system. In the cyclic voltammetric analysis of AA, the initial voltammograms of AA in 67% ethanol were taken over a wide potential range of −1.5 to +1.5 V to locate the reduction/oxidation waves (data not shown). We then elected to focus on the potential window in which the reversible electrochemistry occurs, i.e. −0.2 to −0.5 V. The electrochemistry of AA in salt and buffer was determined at pH 7 because it is physiologically relevant. The potential of the glassy carbon electrode was scanned positively from −500 to −200 mV and back at varying scan rates. Cyclic voltammograms (Fig. 2a) showed a well-defined cathodic peak current for reduction and anodic peak current for oxidation that is characteristic of a reversible redox process. At a scan rate of 100 mV s$^{-1}$, the cathodic peak was at −360 mV and the anodic peak was at −327 mV. The formal reduction potential was −343.5 mV versus Ag/AgCl, and the potential peak separation ($\Delta E_p$) was 33 mV. The $\Delta E_p$ is consistent with a reversible two-electron transfer process that is ideally 29 mV. A plot of cathodic and anodic peak currents versus the square root of scan rate in Fig. 2(b) yielded a straight line, which is characteristic of a diffusion-controlled reversible electrochemical reaction.

**Spectroscopic analysis of AA**

To further characterize AA, its spectroscopic properties were evaluated by UV–Vis and emission spectroscopy to compare its spectrum with that of PYO. Due to the limited sample, AA was diluted in 0.1 M phosphate buffer (pH 7.0) at a ratio of 1:20 and its absorption spectra were recorded. Three distinct absorption peaks were obtained at 276, 398 and 512 nm (Fig. 3a). PYO has shown different absorption peaks at 311 and 381 nm and a very characteristic peak at 690 nm (Reszka et al., 2012). Absorption spectroscopy was used to determine the concentration of AA obtained by chromatography. The absorbance value of 0.45 from the broad peak at 516 nm (visible region) was chosen to determine the concentration of AA to avoid absorbance from interference that usually occurs at lower wavelengths. The Beer–Lambert equation, $A = abc$, was used to calculate the concentration of AA at a path length of 1 cm using an extinction coefficient ($\varepsilon$) of 14 125 M$^{-1}$ cm$^{-1}$, which was derived from the value of log $\varepsilon$ of AA at 515 nm at pH 6.95 in 0.1 M phosphate buffer (Holliman, 1969). The concentration of AA was calculated as 32 µM. The emission spectroscopy analysis of AA was performed on the same sample of AA used for the absorption spectroscopy above.
The biological function of all pigments in *P. aeruginosa* is unclear. Therefore, we assessed where AA was produced during a normal aerobic growth cycle similar to PYO. In wild-type bacteria, Fig. 5(a) shows that AA is produced maximally after a minimum of 24 h growth in LPSM whereas PYO production was maximal at 24 h growth. When we maximized production of AA by inactivation of the *phzS* gene, thereby not allowing for PYO production, we showed that AA production was maximal at 23 h of growth (Fig. 5b). Interestingly, with AA accumulation over time by the *phzS* mutant, we observed a significant decrease in cell density. In contrast, the wild-type strain did not exhibit a similar growth pattern upon production of PYO, although both strains grew to their maximal optical density after ~17 h in LPSM (PAO1 OD$_{750}$ of 0.849 versus the *phzS* mutant OD$_{750}$ of 0.524) prior to the marked decrease in density of the *phzS* mutant over time. Finally, we evaluated the role of QS in the production of AA. In Fig. 5(c) (top row, 1 day of growth), note that the *P. aeruginosa rhlI* mutant does not produce PYO (blue chloroform-extractable pigment) or AA (reddish water-soluble pigment) after 2 days’ growth. At least partial restoration was mediated by the addition of PAI-2 to the *rhlI* mutant (see top and lower two panels of this strain in particular). Addition of PAI-1 to the *lasI rhlI* mutant allowed for overproduction of both PYO and AA because the *rhlI* gene is still intact so that the LasR–PAI-1 complex can activate the rhl QS system, thereby allowing for production of PYO and AA. However, AA production was eliminated in the *lasI rhlI* strain when PAI-1 and PAI-2 were added to the system, probably a result of forcing the complete phenazine pathway toward the production of PYO.

**Controlled potential electrochemistry of AA**

AA clearly exhibits reversible electrochemistry that is evident from the cyclic voltammetry results. Spectro-electrochemical experiments were performed on a drop of sample to determine the effect of redox states on the potential fluorescent properties of AA. A 0.64 mm thick sample layer was sandwiched between the glassy carbon working electrode and the hydrophobic microscope slide as the auxiliary and reference electrodes touched the sample drop from the sides. AA was excited at 441.6 nm at an angle of 90° to the source of the radiant energy and its fluorescence was measured. The potential of the glassy carbon electrode was then held constant at −500 mV for 13 min to completely reduce AA, and fluorescence and electrolysis were monitored simultaneously. Fig. 4 shows the emission spectra with no electrolysis, at the beginning and at the end of the electrolysis. As the sample was reduced, the intensity of the 606 nm emission peak slowly dissipated and eventually disappeared after 13 min. This signifies that AA possesses its largest fluorescent peak in its oxidized form, even before electrochemistry is initiated. Fluorescence then decreased as it was reduced at the negative potential of −500 mV.

**Production of AA is controlled by QS and is produced maximally in the stationary growth phase**

The biological function of all pigments in *P. aeruginosa* is known with the exception of AA, outside of its role as a substrate for a PYO-generating FAD-dependent monooxygenase (Mavrodi *et al.*, 2001). A PubMed search of the word ‘pyorubrin’ recovered only four hits, as virtually nothing is known of the biology of this phenazine pigment and only about six papers have been published on AA to date to our knowledge (Blazevic *et al.*, 1973; Kandela *et al.*, 1997; Loiseau-Marolleau, 1977; Ogunnariwo & Hamilton-Miller, 1975; Palumbo, 1972; Propst & Lubin, 1979). While it is well known that the production of the most notorious toxic phenazine pigment PYO is controlled by the intercellular signalling system known as QS (Brint & Ohman, 1995), the precise timing of AA production by *P. aeruginosa* is unclear.

![Emission spectra (excitation 441.6 nm) of 32 mM AA in 0.05 M phosphate buffer and 0.1 M NaCl at glassy carbon vs. Ag/AgCl electrodes at a potential of −500 mV for 13 min of electrolysis (...) and after 13 min of electrolysis (---).](http://mic.sgmjournals.org/1741)

**Role of AA in bactericidal and cytocidal activity**

Because of the redox properties of AA described above, we next tested for potential antibacterial activity against *E. coli* DH5–*z* and a methicillin-resistant *S. aureus* strain, USA300. These bacteria represent examples of Gram-negative and Gram-positive organisms, respectively, to test for susceptibility or resistance. Using a classical filter paper disc diffusion agar assay (e.g. Kirby–Bauer test; Bauer *et al.*, 1959), AA showed no antimicrobial activity against either organism even at concentrations of 1 mM (Fig. 6a, b, and data not shown). As expected, and serving as a positive antimicrobial control, PYO clearly possessed microbicidal activity against both *E. coli* (Fig. 6a) and *S. aureus* (Fig. 6b) (Reszka *et al.*, 2012). We also tested the effects of AA on human keratinocytes. The results in Fig. 6(c) clearly show...
that AA (pink bars) has no adverse effects on the viability of these cells. This was in contrast to the brown pigment (yellow bars) that showed a concentration-dependent effect on the killing of keratinocytes relative to control keratinocyte medium (Ker media) and the 10% formaldehyde control (violet bar labelled ‘fixed’ in Fig. 6c).

**Fig. 5.** Kinetics of AA production in (a) wild-type vs. (b) phzS mutant of *P. aeruginosa* grown in aerobic LPSM for 28 h. (c) Effects of insertional inactivation of *lasI*, *rhlI* and *lasI rhlI* on pigment production in bacteria grown aerobically in LPSM after chloroform extraction of 1 or 2 day culture supernatants [top layer, aqueous AA fraction; bottom layer, PYO (chloroform) fraction]. The final concentrations in solution of PAI-1 and PAI-2 were 2 and 3.8 μM, respectively.
DISCUSSION

History and biosynthesis of AA in *P. aeruginosa*: clues to produce and optimize its synthesis

The production of various pigments by *P. aeruginosa* has been recognized by scientists and clinicians alike since the late 1800s (Flugge, 1886). In fact, one of the diagnostic features of *P. aeruginosa* infections in various wound dressings (e.g. third degree burns (Holder, 1983) and in CF sputum (Yabuuchi & Ohyama, 1972) is a bluish tinge, indicative of the production of PYO and potentially other pigments, which could include AA. Red-pigmented *P. aeruginosa* strains have also been isolated in ~3.5 % of strains during urinary tract infections in one study (Brown & Lowbury, 1965), 6 % in another (Phillips, 1969) and up to 30 % on *Pseudomonas* agar in strains that did not produce the brown/black pigment pyomelanin (Yabuuchi & Ohyama, 1972).

Based upon a recent PubMed search, there are only six relatively obscure papers that address the ‘red’ pigment(s) of some *P. aeruginosa* strains (Blazevic et al., 1973; Kandela et al., 1997; Loiseau-Marolleau, 1977; Ogunnariwo & Hamilton-Miller, 1975; Palumbo, 1972; Propst & Lubin, 1979). The synthesis of AA started with very simple yet revealing experiments. For example, using only a single amino medium, AA was detected predominantly using L-α-alanine and β-alanine (Yabuuchi & Ohyama, 1972). Glutamate also appears to be optimal for AA production in a minimal salts medium (Ogunnariwo & Hamilton-Miller, 1975). Sulfur sources, in particular L-methionine, L-cysteine and thioglycollate, were also deemed critical for optimal production of AA. In this study, we simplified the media by using LPSM, a medium that we have previously used in other studies to overproduce PYO (Hassett et al., 1992; Reszka et al., 2012). However, in the *phzS* mutant strain (Mavrodi et al., 2001), PYO cannot be formed due to the lack of an FAD-dependent monooxygenase, thereby forcing overproduction of AA (Fig. 1b).

AA from a red-pigmented strain of *P. aeruginosa* was isolated by Holliman (1969) and defined to be the inner salt of 2-amino-6-carboxy-10-methylphenazinium hydroxide. Saha et al. (2008) performed a very preliminary analysis of what was deemed purified AA, but there was no clear evidence that the preparation was truly pure. In fact, the simple procedure of chloroform extraction is not the...
sole criterion for chemical purification of AA as used in their studies. Gibson et al. (2009) also reported the purification of AA from P. aeruginosa using classically elegant chromatographic procedures. Here we elected to use a genetic, biochemical and biological experimental platform involving (i) the use of a phzS mutant (that forces overproduction of AA under phosphate limitation), (ii) TLC (that revealed that the water-soluble ‘red’ pigment is really a compilation of pigments) and finally (iii) MS analysis of each of the purified product(s).

**Synthesis and chemical properties of AA**

A biochemical scheme for PYO biosynthesis in P. aeruginosa had been proposed by Byng et al. (1979), who performed studies on the pigmentation patterns of three classes of mutants incapable of PYO production and a powerful inhibitor, p-aminobenzoate. One group of mutants (group I) produced phenazine-1-carboxylic acid, phenazine-1-carboxamide, AA and aeruginosin B. The aeruginosins were produced biochemically from a proposed branch point intermediate, 5MPCA, and m-aminobenzoic acid (Hansford et al., 1972), the former of which was proven to be a biological precursor for both PYO and AA in vitro and in vivo (in various P. aeruginosa isolates) (Byng et al., 1979; Hansford et al., 1972). Thus, the betaine intermediate appears to be either highly reactive or labile in vivo, because it could not be isolated from various AA-producing P. aeruginosa strains (Byng et al., 1979; Hansford et al., 1972). Once the entire P. aeruginosa genome became available to the public, Mavrodi et al. (2001) used an elegant genetic approach describing a detailed cloning and functional analysis of genes for a phenazine biosynthetic pathway in P. aeruginosa PAO1. They identified two homologous loci responsible for synthesis of phenazine-1-carboxylic acid, and three additional genes (phzM, phzS and phzH) encoding unique enzymes involved in the conversion of phenazine-1-carboxylic acid ultimately to pyocyanin and/or phenazine-1-carboxamide (Mavrodi et al., 2001; Fig. 1a). They assumed that the phzS knockout strain resembled the group I mutants obtained by Byng et al. (1979) and found that the phzS mutant produced large amounts of a dark red water-soluble pigment, which was extremely hydrophilic and could not be identified conclusively due to failure to extract the pigment with the solvents commonly used to recover phenazines. Thus, Mavrodi et al. (2001) hypothesized that the dark red pigment synthesized by the phzS mutant was not AA and aeruginosin B, but rather a coloured intermediate(s) that they believed to be 5MPCA. However, we found that such a red pigment(s) produced by the phzS mutant was actually a mixture of three compounds (AA, phenazine-1-carboxylic acid and an unknown brown pigment) by TLC and MS analysis (Fig. 1b–d). Our data supported the earlier observation of pigmentation pattern of the group I mutants by Byng et al. (1979). Unfortunately, we were unable to resolve the chemical structure of the brown compound, which had reversible electrochemistry (−200 to −620 mV), a broad absorbance band at no distinct peak and significant fluorescence at 605 nm (data not shown). The compound exhibited none of the characteristics of phenazine-related compounds, thus ruling out the presence of either phenazine-1-carboxamide or 5MPCA. MS analysis indicated that the brown pigment might probably be a melanin-related compound, but further experimentation is required for accurate identification.

Phenazine dyes in their monomeric and polymeric states are known to be electroactive, capable of being excellent redox mediators for sensor and biosensor development (Pauliukaitė et al., 2010). Electrochemical studies of some phenazines such as PYO, phenazine-1-carboxylate, phenazine-1-carboxymide and 1-hydroxyphenazine resulted in negative formal reduction potentials at pH 7 (Wang & Newman, 2008). Our cyclic voltammetric analyses indicate clear redox properties of AA with a negative formal reduction potential at pH 7. Generally, phenazines are reduced by two-electron, two-proton transfer processes (Price-Whelan et al., 2006). To our knowledge, this is the first report of the formal reduction potential of AA in the literature in phosphate buffer (pH 7) and supporting electrolyte solution. Phenazines also possess colours due to their aromatic core and these colours differ in the functional group(s) that surround the core. Results from UV-Vis spectroscopy of PYO, the downstream product of AA, in an unbuffered aqueous solution at pH 6.3 showed absorption maxima at 239, 312, 379 and 690 nm (Vukomanovic et al., 1997). AA possessed absorption maxima within the same range for PYO but specifically at 276, 398 and 512 nm.

**A possible rationale for AA production during disease: role of QS**

Unlike AA, PYO is a known omnipotent toxin that kills bacteria, fungi and human cells (Fig. 6c, yellow bars; for a review, see Lau et al., 2005). Many bacteria produce pigments that are/may be considered contributors to virulence similar in context to PYO. These include melanin (black, Cryptococcus neoformans; Nurudeen & Ahearn, 1979), prodigiosin (red, Serratia marcescens; Fineran et al., 2007), porphyrin (black, Porphyromonas gingivalis; Lewis, 2010), staphyloxanthin (yellow/gold, S. aureus; Liu et al., 2005), granadaene (orange/red, Streptococcus agalactiae; Liu et al., 2004), and, among several others that are not mentioned here due to space issues, haemoglobin (brown/black, Plasmodium spp.; Sherry et al., 1995). These pigments offer a variety of functions including antioxidant properties, cytotoxicity, pro-inflammatory properties, ciliary stasis and antimicrobial penetration blockage (for a review, see Liu & Nizet, 2009).

Thus, PYO production due to phosphate deprivation and adequate iron levels (Cox, 1986; Hassett et al., 1992) allows the bacteria to compete in soil and aqueous settings (lakes, rivers, streams, estuaries, etc.) or during infection of
immunocompromised patients against phagocytic cells (e.g. neutrophils). Ramóš et al. (2010) have also revealed another previously unknown property of PYO using a flow cell biofilm system, and showed that the addition of PYO (not AA) influenced swimming motility and the surface-to-volume ratio of mature biofilms. PYO cannot be produced without PhzS and PYO production is controlled by the rhl QS circuit (Brint & Ohman, 1995). Fig. 5 demonstrates that production of AA is QS-dependent and accordingly is optimal during the stationary phase of growth during aerobic conditions but not under anaerobic conditions (data not shown). However, the artificial nature of the shake flask does not tell the true tale of when the organism produces AA, nor does it shed any light on the role of this pigment from a chemical and biological perspective.

In CF airway disease, some patients harbour P. aeruginosa strains that produce predominantly reddish pigments, probably consisting of a mixture of AA and other red or reddish-brown pigments (Fig. 6 and Fig. 1b). We offer a rationale for such behaviour. For example, were PYO to be produced in copious amounts, as it is in CF and other forms of chronic P. aeruginosa diseases [CF (Wilson et al., 1988), chronic obstructive pulmonary disease (Abusriwil & Stockley, 2007), bronchiectasis (Wilson et al., 1988)], this would trigger the immune system to send professional phagocytes to kill P. aeruginosa but also harm airway cells. However, organisms secreting AA would probably not trigger such a response. In fact, we have exposed human keratinocytes to AA and it was not toxic to these cells (Fig. 6, pink bars). However, a brown, water-soluble compound showed some toxicity to human keratinocytes (Fig. 6, yellow bars). Thus, we believe that there are mutations that occur in vivo that foster AA production during the process of CF and probably the aforementioned airway diseases, and there are clear and distinct genetic modifications that occur with time, especially during chronic infection. In CF, many strains have been discovered that actually are incapable of QS (Schaber et al., 2004, 2007). Such strains would not produce PYO and AA. The rationale for this would be that such organisms would not secrete pro-inflammatory virulence factors that are controlled by the rhl QS circuit (for a review, see Hassett et al., 2002) as there is evidence that both the two powerful first- and second-tier P. aeruginosa QS regulators can be mutated during CF airway disease (Hoffman et al., 2009; Schaber et al., 2004).

Recently, phenazine 5MPCA and its analogue, PMS, were reported to induce red pigmentation and death of Candida albicans, and formation of red pigmentation directly correlated with decreased fungal viability (Gibson et al., 2009; Morales et al., 2010). Further experimental analyses demonstrated that PMS can be covalently modified by amino acids within the fungus, and such modifications yield cell-bound red phenazine derivatives, which can generate reactive oxygen species due to their unaltered redox activity. An antifungal mechanism was proposed in which the permanent localization of these red methylphenazinium derivatives within the cell allows for the constant generation of toxic levels of reactive oxygen species and, together with other consequences of modification of cellular macromolecules, leads to efficient fungal killing (Morales et al., 2010). In contrast, PYO was not modified by Candida albicans, did not accumulate within the fungal cells and was less toxic than 5MPCA (Morales et al., 2010). Since Candida albicans is frequently isolated from sputum cultures of CF patients (Chotirmall et al., 2010), phenazine 5MPCA may provide an advantage for P. aeruginosa in competing with or even controlling fungal populations in the CF airway. Interestingly, as one 5MPCA-derived red soluble pigment, AA showed no antibacterial against E. coli or S. aureus (representatives of Gram-negative and Gram-positive organisms or cytoidal activity; Fig. 6), nor was it toxic to Candida albicans (Gibson et al., 2009). Similar to AA, we have also shown that airway peroxidases [lactoperoxidase (LPO)/myeloperoxidase (MPO)] in combination with H₂O₂ and NaNO₂ generate a green nitrated form of PYO that is also non-toxic to E. coli and S. aureus, which are highly susceptible to unaltered PYO (Reszka et al., 2012).

A potential application for AA for human use

Could there be a practical use for AA in humans? Recall that nearly two-thirds of prescribed antibiotics are actually produced by micro-organisms, such as members of the genus Streptomyces, which can have antifungal, antibacterial and antiparasitic properties. The cosmetic industry typically uses a variety of pigments, either solely or in combination, for products including lipstick, nail polish, rouge and mascara. For thousands of years, even in prehistoric times, haematite (or red ochre) was used in cave paintings, body art, cosmetics, and even lipsticks. Cinnabar in its ground form is the pigment vermilion and this pigment was originally used in combination with other pigments to create red paint. Cinnabar is frequently isolated from sputum cultures of CF patients (Chotirmall et al., 2010). In contrast, PYO was not modified by C. albicans, did not accumulate within the fungal cells and was less toxic than 5MPCA (Morales et al., 2010). Since C. albicans is frequently isolated from sputum cultures of CF patients (Chotirmall et al., 2010), phenazine 5MPCA may provide an advantage for P. aeruginosa in competing with or even controlling fungal populations in the CF airway. Interestingly, as one 5MPCA-derived red soluble pigment, AA showed no antibacterial against E. coli or S. aureus (representatives of Gram-negative and Gram-positive organisms or cytoidal activity; Fig. 6), nor was it toxic to C. albicans (Gibson et al., 2009). Similar to AA, we have also shown that airway peroxidases [lactoperoxidase (LPO)/myeloperoxidase (MPO)] in combination with H₂O₂ and NaNO₂ generate a green nitrated form of PYO that is also non-toxic to E. coli and S. aureus, which are highly susceptible to unaltered PYO (Reszka et al., 2012).

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brown compound by TLC and MS analysis; (ii) purified AA has characteristic redox, spectroscopic and fluorescent properties by cyclic voltammetric and spectro-electrochemical analyses; and (iii) stationary-phase production of the AA fraction of the phzS mutant is controlled by the rhl QS circuit. Finally, AA exhibited no antibacterial (Gram-negative or Gram-positive) or cytotoxic activity under the conditions used in this study.

ACKNOWLEDGEMENTS

Support was provided by flexible funds to D.J.H. from the Department of Molecular Genetics, Biochemistry and Microbiology at the University of Cincinnati College of Medicine.

REFERENCES


Edited by: P. Zuber