Investigation of the physiological relationship between the cyanide-insensitive oxidase and cyanide production in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen which demonstrates considerable respiratory versatility, possessing up to five terminal oxidases. One oxidase, the cyanide-insensitive oxidase (CIO), has been previously shown to be resistant to the potent respiratory inhibitor cyanide, a toxin that is synthesized by this bacterium. This study investigated the physiological relationship between hydrogen cyanide production and the CIO. It was found that cyanide is produced in *P. aeruginosa* at similar levels irrespective of its complement of CIO, indicating that the CIO is not an obligatory electron sink for cyanide synthesis. However, MICs for cyanide and growth in its presence demonstrated that the CIO provides *P. aeruginosa* with protection against the effects of exogenous cyanide. Nevertheless, the presence of cyanide did not affect the viability of *cio* mutant strains compared to the wild-type during prolonged incubation in stationary phase. The detection of the fermentation end products acetate and succinate in stationary-phase culture supernatants suggests that *P. aeruginosa*, irrespective of its CIO complement, may in part rely upon fermentation for energy generation in stationary phase. Furthermore, the decrease in cyanide levels during incubation in sealed flasks suggested that active breakdown of HCN by the culture was taking place. To investigate the possibility that the CIO may play a role in pathogenicity, wild-type and *cio* mutant strains were tested in the paralytic killing model of *Caenorhabditis elegans*, a model in which cyanide is the principal toxic agent leading to nematode death. The CIO mutant had delayed killing kinetics, demonstrating that the CIO is required for full pathogenicity of *P. aeruginosa* in this animal model.

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INTRODUCTION

Pseudomonas aeruginosa is an environmentally ubiquitous opportunistic human and animal pathogen capable of a range of infections, including in the cystic fibrosis (CF) lung as well as nosocomial infections (Lyczak *et al.*, 2000). The broad range of niches inhabited by *P. aeruginosa* is facilitated by a significant nutritional and metabolic flexibility together with the possession of versatile modes of energy metabolism. *P. aeruginosa* is capable of both aerobic and anaerobic respiration as well as fermentation of arginine, although it preferentially obtains its energy from aerobic respiration (Davies *et al.*, 1989; Zannoni, 1989). Recently there has been much interest in the anaerobic respiratory capability of *P. aeruginosa*, in particular the role anaerobic respiration may play in the sticky, dehydrated mucus layer that *P. aeruginosa* inhabits in the CF lung (for a review see Hassett *et al.*, 2002).

Therefore, a full appreciation of the respiratory metabolism of *P. aeruginosa* is important to understanding both its fundamental physiology and the factors significant in its pathogenicity.

P. aeruginosa possesses a branched aerobic electron-transport chain terminated by up to five terminal oxidases (Cooper *et al.*, 2003). Four of these belong to the haem-copper oxidase superfamily, of which two are of the cytochrome *cbb₃* type (Comolli & Donohue, 2002). Another of these oxidases is an *aa₃*-type cytochrome, which is common in many bacterial species, and is most closely related to the mitochondrial cytochrome *c* oxidase (Fujiwara *et al.*, 1992). The *P. aeruginosa* PAO1 genome also contains genes for an uncharacterized haem-copper quinol oxidase related to the *Escherichia coli* cytochrome *bo₃*. The fifth *P. aeruginosa* oxidase is called the cyanide-insensitive oxidase (CIO), and is homologous to the cytochrome *bd* quinol oxidases, which are the only known type of bacterial oxidases that are not members of the haem-copper oxidase family (Cunningham *et al.*, 1997; Jünemann, 1997; Poole & Cook, 2000).

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Abbreviation: CIO, cyanide-insensitive oxidase.

Cytochrome *bd* oxidases have been found in a wide variety of bacteria and most recently have been shown to be present in *Bacteroides fragilis*, previously considered to be a strict anaerobe (Baughn & Malamy, 2004). In many cases cytochrome *bd* oxidases have a very high affinity for oxygen, consistent with a role at low oxygen tensions. However, this is not universally the case; for example, the cytochrome *bd* oxidase of *Azotobacter vinelandii* has a much lower affinity for oxygen than the *E. coli* enzyme, and is induced by high levels of oxygen (D’Mello *et al.*, 1996; Hoffmann *et al.*, 1979; Poole & Cook, 2000). The CIO in *P. aeruginosa*, however, appears not to possess haem *d*, unlike other cytochrome *bd* oxidases; thus the CIO belongs to a class of oxidase which is separate from but related to cytochrome *bd* oxidases (Cunningham *et al.*, 1997). Like cytochrome *bd* oxidases the CIO is relatively insensitive to the potent respiratory inhibitor cyanide, allowing respiration to proceed in the presence of > 1 mM potassium cyanide, whilst oxidases sensitive to cyanide are routinely inhibited by concentrations of the order of 100 µM or lower (Cunningham & Williams, 1995; Poole & Cook, 2000).

Regulation of the CIO is not fully understood, though it is known that it is produced on the entry to stationary phase, and whilst cyanide is a potent inducer of the *cioAB* genes, expression analysis of the *cioAB* genes in a $\Delta hcnB$ mutant has shown that cyanide is not the endogenous inducer (Cooper *et al.*, 2003). There is evidence that the RoxRS two-component system is involved in regulation of the CIO, though the full role of this system has yet to be firmly established (Comolli & Donohue, 2002; Cooper *et al.*, 2003). Mutation and overexpression of the CIO has been shown to result in pleiotropic effects on the cell, including multi-nucleate non-septate filamentation, temperature sensitivity and multiple antibiotic sensitivity (Tavankar *et al.*, 2003).

P. aeruginosa produces an arsenal of virulence factors on entry into stationary phase, including hydrogen cyanide, which is produced at concentrations of around 300 µM (Blumer & Haas, 2000). HCN is made by oxidation of the amino acid glycine and is proposed to occur in two stages via imino acetic acid, but there is no direct evidence for this mechanism as the HCN synthase has not been purified and

its reaction characterized. The purpose of cyanide production by *P. aeruginosa* is far from clear. However, it has been shown that cyanide is the mediating factor in the paralytic killing model of *Caenorhabditis elegans* by *P. aeruginosa*, raising the possibility that this may be significant in the pathogenicity of this bacterium (Gallagher & Manoil, 2001). Additionally, it is known that cyanide can be found in burn wound infections caused by *P. aeruginosa* (Goldfarb & Margraf, 1967).

Cyanide synthesis is coincident with CIO production, although there is no evidence of common regulatory factors and thus the precise relationship between the two has yet to be determined. However, it has been suggested that the CIO could be acting as a sink for electrons produced during the oxidation of glycine to produce cyanide (Blumer & Haas, 2000) and that the CIO could have a role in allowing growth under cyanogenic conditions (Cunningham & Williams, 1995; Cunningham *et al.*, 1997). This paper examines both of these possibilities as well as investigating the contribution of the CIO to pathogenicity in the cyanide-mediated paralytic killing model of *C. elegans*.

METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Strains were grown in LB broth (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹ and 5 g NaCl l⁻¹) at 30 °C with appropriate antibiotic addition. Antibiotics were added as required at the following final concentrations: tetracycline 125 or 300 µg ml⁻¹, carbenicillin 500 µg ml⁻¹ and chloramphenicol 400 µg ml⁻¹ (20 µg ml⁻¹ for *E. coli*). Overnight cultures were prepared by inoculating several colonies into 5 ml LB in a 25 ml sterile universal bottle; 1 ml overnight culture was then used to inoculate 50 ml fresh medium in a 250 ml conical flask, except for growth curves in the presence of exogenous cyanide, where a 250 µl inoculum was used.

Construction of a *cioAB* in-frame deletion mutant. An in-frame deletion of the *cioAB* genes was constructed in *P. aeruginosa* PAO1 using the overlap extension PCR method (Ho *et al.*, 1989) followed by allelic exchange. The primers used were *cioAB*1 (5'-CTAGAGATCTATGTTTCGGATTGGAGGCTATCGAC-3'), *cioAB*2 (5'-CACCAGGACCAGGATCGGCACGTAGGTCAGCAGCGGACCGGT-3'), *cioAB*3 (5'-ACCGGTCCGCTGCTGACCTACGTGCCGATCCTGGTCTCTGGTG 0-3') and *cioAB*4 (5'-CTAGACTAGTGTGATAGCCATCGCCGTGCTTCAC-3'). Two DNA fragments, flanking the

Table 1. Strains

Strain	Genotype	Reference
<i>P. aeruginosa</i>		
PAO1		B. Holloway*
PAO7721	PAO1 Δ <i>cioAB</i> in-frame deletion	This study
PAO6049	<i>met-9011 amiE200 strA</i>	D. Haas*
PAO7701	PAO6049 <i>cio</i> Tet ^r	Cunningham & Williams (1995)
PAO6344	<i>hcnB</i>	D. Haas*
<i>E. coli</i>		
S17-1	<i>recA pro</i> (RP4-2Tet::Mu Kan::Tn7)	

B. Holloway, Monash University, Clayton, Victoria, Australia; D. Haas, Université de Lausanne, Switzerland.

region to be deleted, were generated by PCR using the primer pairs *cioAB1/cioAB2* and *cioAB3/cioAB4*. Complementary extensions were present on the 5' ends of the primers *cioAB2* and *cioAB3*. The two PCR fragments generated were mixed and used as templates for the primers *cioAB1* and *cioAB4* to generate a fragment that contained the required deletion. The deletion fragment, *cioAB1/cioAB4*, was then introduced into the *Bgl*II and *Spe*I restriction sites of the suicide plasmid pDM4 (Milton *et al.*, 1996) using *E. coli* strain S17-1, and the plasmid was introduced into PAO1 by conjugation. Allelic exchange of the wild-type *cioAB* region with the *cioAB1/cioAB4* fragment was achieved by double homologous recombination using the *sacB*-based sucrose-selection technique as previously described (Milton *et al.*, 1996). This resulted in an in-frame deletion of 1875 bp between nucleotide position 300 of the *cioA* gene and nucleotide position 705 of the downstream co-transcribed *cioB* gene, which was confirmed by PCR and DNA sequencing analyses.

RT-PCR. To determine whether the in-frame deletion mutation in *cioAB* was non-polar, primer pairs were designed to amplify the *cioAB* transcript alone and a putative transcript comprising part of *cioB* and the ORF immediately downstream, PA3928. These primer pairs are *cioABrightflankF* (5'-GCTCACCTGGTGTGATCT-3')//*cioABrightflankR* (5'-CGCGGAACACGTAGTAGCTC-3') and *cioABrightflankF/PA3928R* (5'-CATGAACAGGCGCATCAG-3'). RNA was extracted from stationary-phase cultures using TRIzol (Invitrogen) and DNase (Invitrogen) treated according to the manufacturers' instructions. Triplicate samples were harvested for each strain. RT-PCR was carried out using the Qiagen OneStep RT-PCR kit. Genomic DNA contamination of the harvested RNA was controlled for all samples by performing a PCR reaction omitting the reverse transcriptase step from the Qiagen OneStep RT-PCR reaction. In all reactions *P. aeruginosa* genomic DNA was used as a positive control.

Cyanide measurements. Cyanide levels were determined using a cyanide ion-selective electrode (Zlosnik & Williams, 2004).

Cyanide minimum inhibitory concentrations. Minimum inhibitory concentrations (MICs) for various strains were determined by twofold dilutions of cyanide in 5 ml LB broth in a 25 ml screw-top Sterilin tube containing any relevant antibiotics. Approximately 5×10^6 cells of diluted mid-exponential-phase culture (OD_{600} 0.7) or stationary-phase culture (OD_{600} 1.7) were inoculated, equating to a final concentration of 1×10^6 cells ml⁻¹. These cultures were then incubated overnight at 30 °C in a shaking incubator at 200 r.p.m. and analysed by eye to determine the presence or absence of growth.

Fermentation end product analysis. Cultures were grown for 3 h into stationary phase (9 h from the start of the culture) in a flask stoppered with a foam bung; this was then replaced with a 'Suba seal' rubber bung. Flasks were harvested at 3, 52 and 125 h into stationary phase; supernatant fractions were prepared by centrifugation of 15 ml of culture at 3200 g for 25 min at 4 °C and stored at -80 °C prior to analysis. Metabolite concentrations in the growth medium were measured by ¹H NMR spectroscopy. A 600 MHz Bruker Avance DRX600 spectrometer was used, equipped with a 5 mm broad-band inverse probe. Immediately prior to analysis the samples were cleared by centrifugation (10 min, 16 000 g), and 790 µl mixed with 200 µl ²H₂O buffer (0.1 M phosphate, pH 7.0) also containing 0.6 mM sodium trimethylsilyl-2,2,3,3-tetradutero-propionate (TSP) and 10 µl 2% NaN₃ solution. This was then mixed, centrifuged again, and 550 µl transferred to an NMR tube. The ²H₂O provided a field-frequency lock for the spectrometer, and the TSP served as an internal chemical shift reference. Spectra were acquired at 300 K using a NOESYPRESAT water-suppression pulse sequence. The spectra were acquired across a 12 kHz spectral width into 32K data points, giving an associated acquisition time of 1.36 s, with an additional T1 relaxation recovery delay of 6 s. Four dummy scans were followed by 128 transients; the summed data were then

multiplied by an exponential apodization factor equivalent to 0.5 Hz line broadening, and Fourier transformed. The spectra were then phased and baseline-corrected using ACDLabs 1D NMR Processor 7.0. The presence of target metabolites (ethanol, lactate, acetate, pyruvate, succinate, ornithine) was then determined based on their known chemical shifts (Lindon *et al.*, 1999). Signals were integrated manually and expressed relative to the TSP resonance.

***C. elegans* paralytic killing experiments.** The *C. elegans* strain used in this work was wild-type Bristol strain N2. Nematodes were grown at 22–23 °C on NG agar with *E. coli* OP50 as a food source and were handled using standard techniques (Brenner, 1974; Wood, 1988). Paralytic killing assays were carried out using as described by Gallagher & Manoil (2001).

RESULTS

Construction of a *cioAB* in-frame deletion mutant

All previously published studies on the CIO have used insertion mutants, either with a miniD-171 insertion or with a gentamicin cassette. Such mutants may cause polar effects on downstream genes. Therefore, an in-frame deletion of 1875 bp, which corresponds to most of the coding sequence of the *cioA* and *cioB* genes, was constructed in the PAO1 background, generating the mutant strain PAO7721. The mutation of *cioAB* was confirmed by PCR and DNA sequencing. Phenotypic analyses confirmed loss of CIO activity in the PAO7721 mutant as determined by failure to grow on 1 mM sodium azide plates and by lack of CIO activity measured in a Clark-type oxygen electrode (data not shown, Cunningham & Williams, 1995).

The in-frame deletion mutant would be expected to be non-polar, but to confirm this we carried out an RT-PCR experiment. We designed primers to amplify a *cioB* transcript downstream of the deletion, and a putative *cioB*-PA3928 transcript. PA3928 is a 171 bp putative ORF immediately downstream of *cioB*. In both wild-type and *cio* mutant samples, primers designed to *cioB* downstream of the deletion in PAO7721 successfully amplified a product, whilst primers designed to include both the remainder of the *cioB* transcript and any co-transcribed PA3928 did not produce a product (data not shown). This demonstrates that the in-frame deletion mutant of *cioAB* is non-polar and furthermore allows us to conclude that the *cioAB* genes are not co-transcribed with PA3928.

Cyanide is produced at equivalent levels in stationary phase, irrespective of the CIO complement of the cell

To test the hypothesis that the CIO acts as a sink for electrons produced during cyanogenesis (Blumer & Haas, 2000), a range of *P. aeruginosa* strains were tested for cyanide production in stationary phase. These data show that in all strains, whether mutant in the *cio* gene, containing wild-type *cio*, or overexpressing *cio*, cyanide is produced at broadly equivalent levels (Fig. 1). A *t*-test showed that there was no significant difference between isogenic strains PAO1 and

Fig. 1. Cyanide production in liquid culture of strains of *P. aeruginosa*. Cyanide was measured in the culture supernatant from a culture 3 h into stationary phase. Measurements are the mean $\pm$ SD of three independent replicates. *No detectable cyanide.

PAO7721 ($P=0.075$) nor between PAO6049 and PAO7701 ($P=0.365$) and therefore mutation of the *cio* genes does not result in any detectable difference in cyanide production. In all cases a mutant deficient in cyanide production (*hcnB*) did not produce any detectable cyanide. The levels of cyanide assayed are in agreement with previous observations of cyanide production (Pessi & Haas, 2000). The presence of wild-type levels of cyanide production in mutants from two separate isogenic pairs (PAO1/PAO7721 and PAO6049/PAO7701) demonstrates that the CIO cannot be acting as the sole sink for electrons produced during cyanogenesis.

Cyanide MICs demonstrate that the CIO does provide *P. aeruginosa* with protection against exogenous cyanide

The synthesis of wild-type levels of cyanide in stationary-phase cultures of CIO mutants leaves open the question of the role of the CIO in relation to cyanide production in *P. aeruginosa*. Previous data have shown that the CIO does permit oxygen uptake in membrane preparations of *P. aeruginosa* in the presence of levels of cyanide that are inhibitory to other terminal oxidases. This suggests that the CIO could have a role in providing tolerance to cyanide. To examine this further, MICs of cyanide were determined. These demonstrate that CIO mutants have cyanide MICs two- to fourfold lower when a stationary-phase inoculum is used (Fig. 2). Interestingly, in cultures prepared using an exponential-phase inoculum all strains, except the CIO-overexpressing strain, had equivalent but lower cyanide MICs as compared to those where a stationary-phase inoculum was used.

CIO mutants demonstrate delayed growth in the presence of exogenous cyanide and this is due to outgassing of cyanide from the culture medium

P. aeruginosa strains were grown in the presence of 300 μ M cyanide, around the upper levels of cyanide detected in liquid culture in this study. These experiments showed that

Fig. 2. MICs of cyanide to *P. aeruginosa*. LB medium was inoculated with 5×10^6 cells from either mid-exponential-phase (white bars) or stationary-phase (grey bars) cultures, and cultures were incubated for 18 h at 30 °C. Representative data are shown.

PAO7701 exhibited a significant lag in growth compared to its wild-type parent strain PAO6049 (Fig. 3). This was also seen for PAO7721 compared to its wild-type parent PAO1 (data not shown). The cyanide levels in these cultures dropped over time, reaching less than 100 μ M after 6 h. Therefore, it seems likely that the *cio* mutant starts growth once sufficient cyanide has been removed from the culture to reduce its concentration to non-inhibitory levels. Indeed, loss of cyanide, termed outgassing, from culture medium is a recognized phenomenon in cell culture media (Arun *et al.*, 2005). To test this hypothesis, strains were incubated in the presence of 500 μ M cyanide in flasks sealed with rubber bungs, with foam-bung-stoppered flasks used as a control. This showed clearly that sealing a flask containing cyanide prevented the growth of PAO7701 (Fig. 4a); similar results were also seen for PAO7721 (data not shown). Cyanide measurements in sealed flasks after 24 h incubation also showed that cyanide levels were indeed considerably higher in those incubated with a rubber seal (Fig. 4b).

Effect of cyanide produced by CIO mutants on their viability in stationary phase

The inhibition of growth by cyanide levels equivalent to those produced by *cio* mutants in early stationary phase implies that growth of *cio* mutant strains would be inhibited by cyanide produced on the entry to stationary phase. This is supported by the growth lag observed in fresh liquid culture in the presence of exogenous cyanide at physiologically relevant levels (Fig. 3). Therefore, it is possible that the viability of *cio* mutants in stationary phase is affected by cyanide production. To examine this we looked at the viability of both PAO1 and PAO7721 over 267 h in flasks sealed at the beginning of stationary phase to prevent cyanide release. In this experiment, both mutant and wild-type cultures retained viability (Fig. 5a). Cyanide measurements demonstrated that cyanide remained in the flasks but over the time-course of the experiment levels fell from

Fig. 3. Growth of *P. aeruginosa* strains in 300 μM cyanide. Overnight cultures of (a) wild-type PAO6049 and (b) *cio* mutant PAO7701 were used to inoculate 50 ml LB broth supplemented with 300 μM cyanide. The fresh cultures were incubated at 30 °C and the OD₆₀₀ (■) and cyanide concentrations (◆) were followed. Measurements are the mean ± SD of three independent replicates.

approximately 250 μM at the start to 75 μM at the end (Fig. 5b). As the flasks were sealed this result suggests that cyanide was actively broken down by the stationary-phase cultures. We further attempted to see if *cio* mutants possessed a stationary-phase exit defect; however, in various experiments we were unable to restart exponential-phase growth from stationary-phase cultures under cyanogenic conditions (data not shown).

Because the levels of cyanide present in the flasks exceed the level known to inhibit the haem-copper oxidases in *P. aeruginosa*, the question of how *P. aeruginosa cio* mutants retain viability in the presence of inhibitory levels of cyanide remains. Aerobic respiration would be expected to be

required for maintenance energy generation and for cryptic growth in stationary phase. In the absence of aerobic respiration, energy generation from fermentation might occur and at higher levels in the *cio* mutant. If this were the case then higher levels of fermentation end products would be expected to be present in the *cio* mutant cultures compared to those of the wild-type strain, capable of CIO-mediated respiration. ¹H NMR analysis of culture supernatants for fermentation end products (Table 2) showed the presence of millimolar concentrations of acetate and micromolar concentrations of succinate, both of which are end products of the fermentation of pyruvate (Eschbach *et al.*, 2004). The accumulation of acetate was the largest change observed, i.e. acetate was the major extracellular metabolite

Fig. 4. Cyanide is liberated from flasks during incubation. (a) OD₆₀₀ measurements after 24 h growth at 30 °C in 500 μM cyanide. (b) Cyanide content in flasks after 24 h incubation at 30 °C in 500 μM cyanide. White bars, flasks stoppered with a rubber bung; grey bars, flasks stoppered with a gas-permeable foam bung. Measurements are the mean ± SD of three independent replicates.

Fig. 5. Viability of *P. aeruginosa* over 267 h in flasks sealed on the entry to stationary phase. Strains were grown into stationary phase for 3 h with a foam bung in the flask. The foam bungs were then replaced with rubber seals to prevent loss of cyanide from the flasks and incubation continued. The c.f.u. (a) and cyanide concentrations (b) were then followed for 267 h. ▲, PAO1; ■, PAO7721. Measurements are the mean ± SD of three independent replicates.

produced by the cultures. The acetate was clearly derived from the cultures, as it was not present in LB medium alone (data not shown). The levels of succinate did not change over time, and therefore its presence in the supernatants was most probably due to its presence at low levels in LB medium. Ethanol and pyruvate were not detected in any of the spectra. The resonances of lactate in 1D NMR spectra overlap with those of other metabolites found in LB broth. A 2D COSY (correlated spectroscopy) experiment showed

Fig. 6. Pathogenicity of *P. aeruginosa* in the paralytic killing model of *C. elegans*. ▲, PAO1 wild-type; ■, PAO7721 Δ*cioAB*; ×, PAO6344 *hcnB*; ○, *E. coli* OP50. Measurements are the mean ± SD of three independent experiments.

that LB does contain lactate (data not shown); however, visual inspection of the chemical shift regions where the lactate resonances are found (1.33d and 4.14q) showed no changes, and therefore lactate was neither produced nor consumed by the cells. Additionally, there was no evidence for any production of ornithine, the end product of the ATP-generating arginine deaminase reaction (Vander Wauven *et al.*, 1984), in the supernatant (data not shown). The marked accumulation of acetate suggests that *P. aeruginosa* cultures were fermenting in stationary phase irrespective of their CIO complement.

The CIO is required for full pathogenicity in the paralytic model of *C. elegans* killing

Given that it has been shown that cyanide is the mediating factor in the paralytic killing model of *C. elegans* (Gallagher & Manoil, 2001), we were interested to know what the role of the CIO might be in this model. Using PAO1 and its isogenic CIO mutant, PAO7721, in the paralytic killing model demonstrated that the CIO is required for full pathogenicity (Fig. 6). The CIO mutant has a lag in killing compared to PAO1. For example, after 35 min PAO1 has killed 56 % of the *C. elegans* population, compared to 16 % for PAO7721, whilst after 45 min PAO1 has killed 93 % of

Table 2. Fermentation end products in *P. aeruginosa* culture supernatants

Strain		Time (h)		
		9	58	128
Acetate, mM (±SD)	PAO1	4.59 (0.11)	8.25 (0.18)	8.78 (0.34)
	PAO7721	4.06 (0.58)	8.10 (0.33)	8.80 (0.19)
Succinate, mM (±SD)	PAO1	ND	0.024 (0.0016)	0.031 (0.0018)
	PAO7721	ND	0.026 (0.0012)	0.027 (0.0045)

ND, Succinate levels not determined in 9 h samples because the succinate peak was wholly overlapped by that of another metabolite at that time point.

the *C. elegans* population, compared to 52 % for PAO7721. After 75 min both strains have reached a plateau at 95 % killing for PAO1 and 81 % killing for PAO7721.

DISCUSSION

This study has sought to provide a further insight into the role of the CIO in *P. aeruginosa* and in particular its link with cyanogenesis. Specifically, we addressed two issues: firstly, whether the CIO is the electron sink for the oxidative process of cyanide synthesis from glycine; and secondly, whether the CIO facilitates aerobic respiration of *P. aeruginosa* during cyanide synthesis. It is clear from the data provided here that lack of a functional CIO does not prevent cyanide synthesis (Fig. 1). Strains with different complements of CIO, including mutants and overexpressing strains, showed similar cyanide production levels to the wild-type strains. These data clearly demonstrate that the CIO is not the sole receptor for the electrons produced in cyanogenesis.

Cyanide is a potent respiratory toxin and completely inhibits terminal oxidases at micromolar concentrations, while the CIO is known to be resistant to concentrations greater than 1 mM (Cunningham & Williams, 1995). It is, perhaps, surprising that CIO mutants still produce cyanide at levels that are known to be inhibitory to the other oxidases it possesses. Whilst the CIO has been characterized as resistant to cyanide in activity assays, it has yet to be demonstrated that it physiologically confers cyanide resistance on cultures of *P. aeruginosa*. To address this question cyanide MICs were measured. Using cells from an exponential-phase culture, in which CIO activity is at a minimum (Cooper *et al.*, 2003), all strains had equivalent resistance to cyanide, except the *cioAB*-overexpressing strain, which has increased CIO activity levels (Fig. 2) (Cunningham *et al.*, 1997; Tavankar *et al.*, 2003). However, when cells from a stationary-phase culture were used, in which the CIO activity is maximum, strains possessing a functional CIO had MICs two- to fourfold higher than *cio* mutants (Fig. 2). These data suggest that the CIO does have a role in protecting *P. aeruginosa* from cyanide.

The experiments shown in Figs 3 and 4 provided further evidence of a role for the CIO in relation to cyanide. Mutant and wild-type strains were grown in medium supplemented with 300 μ M cyanide, equivalent to the concentration produced by these strains in batch culture. This experiment is similar to that performed by Comolli & Donohue (2002) on a *cioAB* mutant in a different wild-type background. Our results mirror those seen by Comolli & Donohue (2002), showing that CIO mutants demonstrate a considerable lag in growth (Fig. 3). The kinetics of cyanide loss in culture showed that cyanide is lost sharply until it appears to reach a level after which the *cio* mutants begin to grow (Fig. 3b). This is consistent with the idea that the lag ended as a result of the liberation of cyanide from the flask. This was tested further by inoculating wild-type and *cio* mutant strains into flasks containing 500 μ M cyanide. The CIO mutants did

not grow in sealed flasks, whilst those grown in flasks with gas-permeable foam bungs grew to a similar final optical density as the wild-type strain (Fig. 4). After 24 h incubation the sealed flasks exhibited cyanide concentrations that were higher than that needed to inhibit other terminal oxidases, and furthermore were several times higher than concentrations found in unsealed flasks.

From this work the question arises of how a *cio* mutant can produce wild-type levels of cyanide, despite the fact that growth seems to be inhibited at such concentrations. Cyanide is made upon entry into stationary phase, that is, as culture growth ends, and perhaps this mitigates the toxic effects of cyanide on the *cio* mutants. However, one would predict that aerobic respiration would not be possible in a *cio* mutant strain, so generation of energy for maintenance and cryptic growth in long-term stationary-phase cultures would be compromised and this might affect viability. However, *cio* mutants were not compromised in their stationary-phase viability (Fig. 5a), although they will have difficulty exiting stationary phase when cyanide is present at the levels produced early in this growth phase. It seems clear, from the data presented here, that cyanide is bacteriostatic rather than bacteriocidal and so the question is how *P. aeruginosa* is able to maintain viability given the likely inhibition of its aerobic respiration system. Given that the cultures are grown under aerobic conditions in LB broth without added nitrate, it is unlikely that anaerobic respiration is a factor. However, it is known that *P. aeruginosa* can also obtain ATP both from arginine using arginine deaminase and by the fermentation of pyruvate (Eschbach *et al.*, 2004; Vander Wauven *et al.*, 1984). Indeed Eschbach *et al.* (2004) showed that pyruvate fermentation by *P. aeruginosa* led to enhanced stationary-phase survival under anaerobic conditions. So pyruvate fermentation may be able to provide the energetic needs of *cio* mutants in stationary phase in the presence of inhibitory levels of cyanide; consistent with this is the presence of the fermentation end product acetate in the growth medium (Table 2). However, there was no significant difference in its concentrations between the wild-type and *cio* mutant. Indeed, acetate was present at similar concentrations to those found by Eschbach *et al.* (2004) from *P. aeruginosa* grown anaerobically with 20 mM pyruvate. These data suggest that fermentation is likely aiding long-term viability irrespective of the presence or absence of a functional electron-transport chain. Thus, this provides a possible explanation for the survival the *cio* mutant in the presence of cyanide at levels sufficient to inhibit normal growth and it may be that fermentation provides the electron sink for cyanide synthesis.

Cyanide detoxification may also be a factor in stationary-phase survival of strains. The enzyme rhodanese is made by *P. aeruginosa* and is capable of detoxifying cyanide. It has recently been shown to be capable of providing *E. coli* with additional protection against cyanide when expressed *in trans* (Cipollone *et al.*, 2004, 2006). Indeed data for flasks sealed at stationary phase to prevent loss of cyanide

produced in culture show that the cyanide concentration decreases over time from $\sim 250 \mu\text{M}$ to $\sim 75 \mu\text{M}$, consistent with cyanide degradation by the cultures (Fig. 5b).

In light of the demonstration that cyanide is the mediating factor in the paralytic model of infection of *C. elegans* (Gallagher & Manoil, 2001), we were interested in what role the CIO could be playing in this infection model. There are three different models of *P. aeruginosa*-mediated killing of *C. elegans*: fast killing, slow killing and paralytic killing; the mode of killing depends on the experimental conditions used, principally the medium composition (Darby *et al.*, 1999; Tan *et al.*, 1999). It is known that phenazines mediate the fast killing model whilst cyanide mediates the paralytic killing model, in which rapid neuromuscular paralysis leads to death (Gallagher & Manoil, 2001; Mahajan-Miklos *et al.*, 1999). Given that the CIO provides *P. aeruginosa* with protection against cyanide during active growth it is conceivable that it could be an important adaptation in this pathogenicity model. Our data showed that the *cioAB* deletion mutant was unable to kill *C. elegans* with the same efficiency as the wild-type PAO1 (Fig. 6). Whilst loss of the CIO does not prevent killing of *C. elegans*, as loss of cyanide production does, it is nonetheless clear that the killing efficiency is significantly reduced. Additionally, the *cioAB* mutant failed to achieve 100 % killing. Whilst this is not complete attenuation as seen with the *hcn* mutant (Gallagher & Manoil, 2001) it is nevertheless worth noting that in previous experiments using the fast and slow killing models of *C. elegans*, mutants of the cytotoxic *P. aeruginosa* strain PA14 in *dsbA* (in both models) and *pho34B12* (in the slow killing model) demonstrated similar kinetics of killing, i.e. delayed killing (Tan *et al.*, 1999). Therefore the CIO would appear to be required for full pathogenicity in the paralytic model of *C. elegans* infection. It is conceivable that the delay in killing is due to *P. aeruginosa* having difficulty handling cyanide in the absence of a functioning CIO-terminated electron transport pathway for respiration.

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