Bacterial cyanogenesis occurs in the cystic fibrosis lung.

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The cystic fibrosis lung environment is poorly defined, but data suggest that bacteria may encounter reduced oxygen tensions and possibly a frankly anaerobic environment. *Pseudomonas aeruginosa* produces the potent toxin cyanide under strictly micro-aerobic conditions. We looked for evidence of bacterial cyanogenesis in the CF lung by measuring sputum cyanide concentrations.

Sputum cyanide was measured in seven stable CF patients and before and after intravenous antibiotic therapy during a hospital admission in a further eight patients experiencing acute exacerbations. All patients were chronically infected with *P. aeruginosa*. Comparative sputum data were obtained from nine CF patients with no documented *P. aeruginosa* infection and 10 healthy, non-smoking normal volunteers.

High levels of cyanide were detected in all of the *P. aeruginosa* infected stable CF patients (median 0.56 µg/ml, range 0.37 - 2.81 µg/ml), and in 7/8 acute sputum samples (median 0.73 µg/ml, range 0 – 1.43 µg/ml). In contrast, cyanide was not detectable in sputum from 8/9 CF patients without *P. aeruginosa* infection or in any of the normal controls. Intravenous antibiotic treatment significantly reduced sputum cyanide levels (median 0.73 µg/ml to median 0.0 µg/ml, p=0.05).

The cyanide detected indicates that the CF lung provides a predominantly micro-aerobic environment for *P. aeruginosa*. Cyanide is likely to be a potentially important virulence factor in *P. aeruginosa* infected CF patients.

**Word count: 216**
Pseudomonas aeruginosa is an opportunistic bacterium that causes chronic airway sepsis in individuals with cystic fibrosis (CF) and other destructive lung diseases. In CF, it becomes the predominant pathogen by late childhood. [4] This early acquisition of P. aeruginosa in relatively normal lungs is unique to CF. Much of the lung damage that occurs in CF is thought to be due to ineffective host factors. [2] Bacterial virulence factors undoubtedly also contribute to lung damage, but they have been poorly characterized in vivo. In CF airways, P. aeruginosa is thought to grow in anaerobic/reduced oxygen pockets situated within thickened and tenacious mucus plugs that occlude small airways. [3,4] This environment may be suitable for cyanide production by P. aeruginosa. [20,21] However, at present it is not known whether bacteria are residing in strictly anaerobic or micro-aerobic pockets within the CF lung. P. aeruginosa is one of only a few bacterial species (P. fluorescens, Chromobacterium violaceum and Rhizobium leguminosarum), known to produce cyanide. [3] The P. aeruginosa cyanide synthase enzyme is cell associated and requires molecular oxygen as an electron acceptor. Cyanide is only produced over a very narrow spectrum of environmental oxygen tensions (micro-aerobic) and synthesis is rapidly inactivated under both atmospheric oxygen and strictly anaerobic conditions. [3] We therefore measured cyanide levels in sputum samples from CF patients as both an in vivo marker of P. aeruginosa micro-aerobic growth and to determine if cyanide production could be a potential virulence mechanism in CF lung disease.

METHODS

The Southern Tasmania Health and Medical Human Research Ethics Committee approved the study and all subjects gave written informed consent before participating. Fifteen adult CF patients with chronic P. aeruginosa lung infection (confirmed by repeated routine microbiological testing) were recruited at the time of a routine clinic appointment for stable
patients (≥ one month from most recent exacerbation), or when unwell with worsening cough, breathlessness and sputum purulence and admitted to hospital (acute patients). In the acute patients, a repeat sputum sample was obtained at least one week into the intravenous antibiotic course (median 8.5 days, range 7 – 14 days) at a time point when symptoms were subjectively improving. Fourteen patients were receiving long-term anti-*P. aeruginosa* antibiotic treatment regimes by inhalation (data not shown). One patient was not receiving any sort of antibiotic therapy directed against *P. aeruginosa* at the time of sampling. Nine patients who had not grown *P. aeruginosa* previously also provided sputum samples. Spontaneously expectorated sputum was collected and processed as described previously. [1] Induced sputum samples were obtained from 10 healthy volunteers using the method of Pin *et al.* [1] and processed in the same way. All CF patients and normal controls were current non-smokers.

**Sputum processing**

An aliquot of raw sputum was weighed and an equivalent volume to weight of dithiothreitol (10%) was added and the sample gently vortexed and placed in a water bath (38°C for 30 min). At 10 minute intervals the sample was removed and gently vortexed once again. If the sample appeared particularly tenacious, further mixing was undertaken with gentle pipetting to ensure homogenization. The sample was diluted a further five times with phosphate buffered saline ensuring a constant final dilution effect of ten times and then centrifuged (350 g for 15 minutes). Following centrifugation, the cell free supernatant was decanted off and stored at -80°C in one ml aliquots for later analysis.

**Cyanide detection**
Cyanide was liberated from the processed sputum supernatants by acid treatment and assayed using a standard method (American Public Health Association method 4500-CN) \cite{AmericanPublicHealthAssociation,2001,#516}. The lower limit of detection of the method was 0.05 \text{µg/L}.

**Statistical analysis**

Differences between groups were assessed with the Kruskal-Wallis test for nonparametric data. Wilcoxon's Rank Test was used to compare sputum cyanide levels in samples obtained at the beginning of an exacerbation and then again at the end of an intravenous antibiotic treatment course. A two-tailed p value of 0.05 or less was considered to be statistically significant.

**RESULTS**

Healthy controls were significantly older and had better lung function than the CF patients (Table). Not surprisingly, CF patients without *P. aeruginosa* infection were younger and had better lung function than those with chronic *P. aeruginosa* infection. Cyanide was present in all of the sputum samples from stable CF patients with *P. aeruginosa* (median 0.56 \text{µg/ml}, range 0.37- 2.81 \text{µg/ml}), and in 7/8 samples from exacerbating patients (median 0.73 \text{µg/ml}, range 0.0 - 1.43 \text{µg/ml}). There was no statistical difference between sputum cyanide levels in acute and stable patients (p=0.9). Cyanide was not detected in 8/9 sputum samples from CF patients without *P. aeruginosa* or in any of the sputum samples from normal controls (Table & Figure). The only CF patient without *P. aeruginosa* who had detectable sputum cyanide was under five years of age and this individual has isolated *Staphylococcus aureus* from the only two sputum samples obtained to date.
Intravenous antibiotic treatment for an acute exacerbation reduced sputum cyanide levels (median 0.73 µg/ml, range 0.0 - 1.43 µg/ml to median 0.0 µg/ml, range 0.0 - 1.00 µg/ml, p=0.05). In four of the seven acute patients with detectable cyanide levels on admission, these were reduced to below the detection limit of the assay following intravenous antibiotic treatment.

Interestingly, the highest sputum cyanide concentration was found in a stable patient who was the only individual chronically infected with *P. aeruginosa* not receiving any sort of routine antibiotic therapy directed against this bacterium. There was no relationship between sputum cyanide levels and lung function.
DISCUSSION

This is the first report of cyanide in CF sputum and our results support previous speculation that the CF lung environment may be suitable for *P. aeruginosa* cyanogenesis. Both clinically stable CF patients and those presenting with acute exacerbations of CF had high levels of cyanide, whereas cyanide was undetectable in sputum samples from normal healthy controls and nearly all sputum samples obtained from CF patients from whom *P. aeruginosa* had not previously been isolated. Following intravenous antibiotic treatment for an acute exacerbation, sputum cyanide levels were significantly reduced in patients infected with *P. aeruginosa*.

Cyanide is a very potent poison that causes cell death through irreversible inhibition of mitochondrial oxidative phosphorylation. Cyanide toxicity is well described following smoke inhalational injuries in man, but the role of cyanide in human disease in the setting of *P. aeruginosa* infection is poorly characterized, although cyanide production has been noted in *P. aeruginosa*–infected burn eschars. [1] The cellular toxicity of cyanide is very much influenced by the chemical milieu, but in humans, blood cyanide levels of 0.5 - 1.0 µg/ml are associated with cardiac effects, levels of 2.5 - 3.0 µg/ml result in reduced consciousness and levels greater than 3.0 µg/ml cause death. [4] The sputum levels we detected are therefore well within the toxic range for cell metabolism and will probably be contributing to long-term lung damage in CF.

The capacity of *P. aeruginosa* to produce cyanide has been recognized for some time. Most of our understanding of the genetics, biochemistry and regulation of cyanide production by *P. aeruginosa* comes from the study of its interactions in soil communities where *P. aeruginosa* uses cyanide to protect its niche and eradicate competing organisms. [3] Our finding of high cyanide levels in CF sputum has several disease implications. First, cyanide may be used by *P. aeruginosa* in the lung in the same way that it is utilized in soil, that is, to
protect its niche and this may partly explain why *P. aeruginosa* rapidly becomes the predominant pathogen in CF, apparently displacing other bacterial species. Second, cyanide will be directly toxic to airway cells and finally, cyanide may also contribute to the relative inability of neutrophils to clear *P. aeruginosa* infection in the CF lung. [3,34] Cyanide in the concentrations we report has been shown to inhibit the function of the enzyme myeloperoxidase and prevent production of hyochlorous acid during the oxidative burst. [9] Cyanide may also impair neutrophil migration and cause morphological changes related to damage to the cell cytoskeleton. [4] Interestingly, neutrophils themselves can produce very small amounts of cyanide during chlorination of bacterial cell membranes, [3] but this contribution is likely to be negligible in comparison to the high cyanide levels that we report. Furthermore, we were unable to detect cyanide in 8/9 sputum samples from CF patients not yet infected by *P. aeruginosa*, although they isolated other bacterial pathogens from their sputum and studies have demonstrated that these subjects will also have a florid airway neutrophilia. [4 Pt 1,2] We detected cyanide in sputum from a very young patient who has not isolated *P. aeruginosa* on routine culture. This patient rarely produces sputum and we were fortunate to obtain the sample that we did, but at present we have no explanation for the cyanide detected. There is the potential that the few culture results available from this individual have been false negatives for *P. aeruginosa*, but we have not confirmed this with more invasive techniques. We continue to monitor clinical progress closely and at present the patient appears well.

There has been recent debate on the nature of the microbial habitat in the CF lung, particularly with respect to whether conditions are aerobic or anaerobic. [3,4] Understanding lung environmental conditions is clinically important, as the efficacy of several antibiotics is dependent on the chemical milieu, i.e. the aminoglycosides function very poorly under anaerobic conditions. [10] Our findings suggest that a substantial proportion of the *P.
*aeruginosa* population in the CF lung exists in micro-aerobic (O<sub>2</sub> < 5%), rather than aerobic or anaerobic conditions because *P. aeruginosa* can only produce cyanide under strictly micro-aerobic conditions. [3] Given the oxygen gradient demonstrated in CF mucus it is also possible that there are populations of *P. aeruginosa* dwelling within aerobic or frankly anaerobic regions in the CF lung, however further studies with other metabolic markers are required to confirm this. [3] This habitat diversity may partly explain the refractory nature of infection, as microbial populations in different physiological states may respond quite differently to therapeutic interventions.

In the relatively small number of sputum samples that we tested, cyanide levels were similar whether patients were stable or exacerbating, but levels in the latter patients were substantially reduced by a course of intravenous antibiotics. A valid criticism of the study is that the small numbers of patients we studied has reduced our ability to pick up a difference in sputum cyanide between individuals based on clinical status. Ideally, patients should have been followed from the clinically stable situation into an exacerbation and then been re-assessed at the end of treatment, as well as one month later when stable once again. Such a study clearly needs to be undertaken and correlated with changes in bacterial numbers, confirming a relationship between cyanide and clinical status would open the way to development of tools such as exhaled breath condensates to monitor cyanide as a potential biomarker. Despite the small number of subjects studied, it is interesting to note that the highest cyanide level detected occurred in a stable patient who was the only *P. aeruginosa* infected patient not receiving any form of anti-*P. aeruginosa* therapy. Chronic suppressive inhaled antibiotic treatment strategies directed against *P. aeruginosa* may therefore be partially effective at inhibiting cyanide production, while more aggressive treatment in our study reduced cyanide in the CF lung to undetectable levels in almost half of cases. Thus, routine intravenous treatment courses may be of particular benefit in treating this disease.
Whether the reduction in cyanide levels following in-patient antibiotics was due to alterations in bacterial numbers or to other non-bactericidal effects was not assessed in this study, but it is worth speculating that the excellent outcomes reported in some countries may be due in part to suppression of cyanide production through regular admissions, irrespective of clinical status, for intravenous antibiotic courses. [2,4]

In vitro data suggests that mucoid P. aeruginosa isolates produce more cyanide than non-mucoid laboratory strains, [20] but we did not identify such a relationship in this study as all patients were colonized with mucoid strains (data not shown). Whether strains that produce more cyanide have a survival advantage in the CF lung environment or whether the alginate layer in mucoid strains simply increases cyanide output by limiting oxygen diffusion are questions that need to be further examined.

In summary, this study demonstrates large amounts of cyanide in CF sputum. Cyanide is likely to be an important virulence factor employed by P. aeruginosa in CF and almost certainly will be contributing directly to long-term lung damage and impairment of the local host immune system. Of clinical importance, we show that cyanide production can be reduced to very low levels by aggressive antibiotic therapy. Our findings suggest that a substantial proportion of the bacterial population in the CF lung exists under micro-aerobic rather than anaerobic conditions. Whether cyanide in either sputum or exhaled breath condensates may be a reliable marker of bacterial activity that could be used to assess treatment efficacy in CF requires further study.
References


Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Acute CF patients with <em>P. aeruginosa</em> (N=8)</th>
<th>Stable CF patients with <em>P. aeruginosa</em> (N=7)</th>
<th>+CF patients without <em>P. aeruginosa</em> (N=9)</th>
<th>Normal controls (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>23 (19-48)</td>
<td>23 (17-33)</td>
<td>10 (4-17)</td>
<td>43 (26-63)</td>
</tr>
<tr>
<td>Gender</td>
<td>5F/3M</td>
<td>3F/4M</td>
<td>3F/6M</td>
<td>4F/5M</td>
</tr>
<tr>
<td>Sputum cyanide, µg/ml</td>
<td>*0.73 (0.0-1.43)</td>
<td>*0.56 (0.37-2.81)</td>
<td>0.0 (0.0-0.49)</td>
<td>0.0 (0.0-0.0)</td>
</tr>
<tr>
<td>FEV₁, L</td>
<td>1.55 (0.5-2.84)</td>
<td>1.98 (0.78-4.51)</td>
<td>2.08 (1.06-3.21)</td>
<td>3.57 (2.27-5.12)</td>
</tr>
<tr>
<td>FEV₁ % pred. ** #</td>
<td>**42 (16-67)</td>
<td>**52 (26-98)</td>
<td>** #88 (59-104)</td>
<td>106 (99-127)</td>
</tr>
<tr>
<td>FVC</td>
<td>2.62 (1.00-4.63)</td>
<td>3.02 (1.39-5.50)</td>
<td>2.54 (1.17-4.24)</td>
<td>4.79 (3.06-6.72)</td>
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<td>FVC % pred. ** #</td>
<td>64 (30-83)</td>
<td>72 (40-103)</td>
<td>95 (73-113)</td>
<td>116 (95-141)</td>
</tr>
</tbody>
</table>

* P<0.001; *P. aeruginosa* infected (stable and acute patients) versus CF patients without *P. aeruginosa* infection and healthy controls.

** P<0.01; CF patients versus healthy controls.  # P<0.01; *P. aeruginosa* infected (stable and acute patients) versus CF patients without *P. aeruginosa* infection.

† Lung function results are based on 7 patients; two patients aged four and five years respectively could not manage reproducible spirometry.
Table 2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Sputum microbiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>M</td>
<td><em>Staph. aureus.</em></td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>M</td>
<td><em>MRSA, Sced. prolificans.</em></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>M</td>
<td><em>Staph. aureus, A. fumigatus.</em></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>F</td>
<td><em>Staph. aureus, H. influenzae.</em></td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>F</td>
<td><em>Staph. aureus.</em></td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>M</td>
<td><em>Staph. aureus, Alc. Xylosidans, Sced. prolificans.</em></td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>M</td>
<td><em>A. fumigatus.</em></td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>M</td>
<td><em>Staph. aureus, H. influenzae, A. fumigatus, Sten. maltophilia.</em></td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>F</td>
<td><em>Staph. aureus.</em></td>
</tr>
</tbody>
</table>
Legends

Table 1. Subject demographics. Data are presented as median and range. Lung function in the acute patients represents values achieved within 48 hours of admission. FEV1; forced expiratory volume in 1 second. FVC; forced vital capacity.

Table 2. Demographic details and sputum culture results in stable CF patients without *P. aeruginosa* infection. *Staph. aureus; Staphylococcus aureus*, MRSA; *methicillin resistant Staphylococcus aureus A. fumigatus; Aspergillus fumigatus, Sten. maltophilia; Stenotrophomonas maltophilia, H. influenzae; Haemophilus influenzae. Sced. prolificans; Scedosporium prolificans*, *A. xylosidans; Alcalagines xylosidans. * This patient was the only one with detectable sputum cyanide levels.

Figure. Cyanide concentrations in sputum samples from; CF patients with chronic *P. aeruginosa* infection experiencing an acute exacerbation (n=8), stable patients with chronic *P. aeruginosa* infection (n=7), stable CF patients with no documented *P. aeruginosa* infection (n=9) and healthy controls (n=10). Each point represents one sputum sample.
References


