

## Measurement of *Pseudomonas aeruginosa* Phenazine Pigments in Sputum and Assessment of Their Contribution to Sputum Sol Toxicity for Respiratory Epithelium

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**The phenazine pigments pyocyanin and 1-hydroxyphenazine were resolved by high-pressure liquid chromatography from the sputum sol phase from 9 of 13 patients with cystic fibrosis or bronchiectasis colonized by *Pseudomonas aeruginosa*. The concentrations measured were each sufficient to inhibit ciliary beating in vitro and contributed a significant proportion of sol phase toxicity for respiratory epithelium.**

*Pseudomonas aeruginosa* colonizes the lungs of patients with cystic fibrosis (CF) and other forms of severe bronchiectasis. We have recently demonstrated that pyocyanin and 1-hydroxyphenazine, phenazine redox pigments generated by *P. aeruginosa*, disrupt human ciliary beating in vitro (15). Pyocyanin also inhibits epidermal cell growth (4) and lymphocyte proliferation (7), has antibiotic properties against other microorganisms (9), and influences the acquisition of iron by pseudomonads (3). 1-Hydroxyphenazine inhibits mammalian cell respiration (1).

Sputum was collected from 12 patients who were colonized by *P. aeruginosa*; 8 had bronchiectasis and 4 had CF. Pulmonary secretions, from which *P. aeruginosa* was cultured, were aspirated directly from the large airways of a CF lung removed at transplantation. Sputum was also collected from five patients who had bronchiectasis and who had never been colonized by *P. aeruginosa*. Each sputum sample was centrifuged ( $50,000 \times g$  for 90 min at 4°C), the watery sol phase was retained, and the gel phase was discarded. Each sol phase sample (1 ml) was loaded in 5% acetic acid on a C<sub>18</sub> Sep Pak column (Waters Associates, Inc.) and eluted with 40% propan-2-ol in aqueous acetic acid. The eluant was fractionated by high-pressure liquid chromatography (HPLC) at 2 ml/min on a  $\mu$ Bondapak C<sub>18</sub> column (30 by 0.8 cm) with a 20-min linear gradient from 0 to 40% propan-2-ol in 5% aqueous acetic acid, with monitoring at 280 and 254 nm in UV light (14). Pyocyanin and 1-hydroxyphenazine were identified by their HPLC-UV profiles (14). All pyocyanin concentrations in each sol sample and 1-hydroxyphenazine concentrations in sol samples 1, 2, and 7 were calculated after extraction and full UV analysis; 1-hydroxyphenazine concentrations in other sol samples were calculated from the HPLC-UV peak height.

Human nasal ciliated epithelium was obtained from the inferior turbinate of normal volunteers (8). The sol phase pH was variable (5.5 to 9.5) and was adjusted to 7.4 to ensure that changes in ciliary beat frequency (CBF) were not pH related. A sol phase sample was added to ciliated epithelium, a sealed microscope cover slip slide preparation was constructed, and controls were suspended in phosphate-buffered saline (PBS). Ten epithelium strips were identified, and their positions were marked. CBF was measured in each

epithelial strip by a photometric technique at 1-h intervals for 4 h (8, 15), and a mean was calculated. Any change in beating pattern, ciliary stasis (previously beating cilia subsequently observed to be static), or change in epithelium structure was noted. Results are shown as percent ciliary slowing:  $100 \times [\text{control mean CBF} - \text{lowest mean CBF in the sol phase}] / \text{control mean CBF}$ . The slowest mean CBF of the test preparation was identified and compared with the control mean CBF at the same timepoint by the unpaired *t* test (20 readings, 18 degrees of freedom). These experiments were repeated with sol phase samples after chloroform extraction and after the addition of sufficient  $\alpha_1$ -antitrypsinase (5 mg/ml) to inhibit all elastase activity, as measured by a fluorescein-elastin technique (12).

Each sol phase sample (1 ml) was processed and fractionated by HPLC as previously described. HPLC fractions containing the phenazines were collected, quantified by UV absorbance measurements and, in some cases, fully characterized by mass spectrometry (14). The fractions were dried under vacuum and then reconstituted in PBS (1 ml), and their effect on CBF was assayed. In addition, all HPLC fractions obtained from the chloroform extract of the sol phase from patient 1 (see Table 1) were similarly assayed.

The sol phase caused significant ( $P < 0.001$ ) ciliary slowing in nine cases and ciliary dyskinesia (loss of the normal coordinated pattern of ciliary beating), ciliary stasis, and epithelial disruption (loss of the integrity of the epithelial surface, which became broken and irregular) in eight cases. Pyocyanin and 1-hydroxyphenazine were detected in 9 of the 13 sol phase samples from patients colonized by *P. aeruginosa* (Table 1) but were undetectable in the 4 sol phase samples which did not affect ciliary beating. The level of 1-hydroxyphenazine was higher in the sample aspirated directly from the large airways of a resected lung than in sputum. We have previously shown (15) that pyocyanin (4  $\mu\text{g/ml}$ ) causes a gradual slowing of CBF over 4 h associated with epithelial disruption, that pyocyanin (0.2  $\mu\text{g/ml}$ ) causes similar changes over 24 h, and that 1-hydroxyphenazine (2  $\mu\text{g/ml}$ ) has an immediate action and causes ciliary slowing and dyskinesia. In the present study the concentration of pyocyanin in sputum was often in excess of that previously demonstrated to have a significant effect on CBF in vitro (15). The concentration of 1-hydroxyphenazine, although lower, was also sometimes sufficient to cause significant

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TABLE 1. Concentrations of phenazines in sputum sol phase samples and effects of sputum sol samples on human CBF

Sputum sol sample (disease state) <sup>a</sup>	Concn (μg/ml) in sputum sol phase of:		% Ciliary slowing during a 4-h experiment		
	Pyocyanin	1-Hydroxyphenazine	In sputum sol sample	After chloroform extraction	After α <sub>1</sub> -antiproteinase treatment
1 (B)	27.3	3.5	100	4	61
2 (B)	19.0	1.0	100	21	85
3 (B)	2.5	0.1	100	8	100
4 (B)	1.7	0.5	100	18	100
5 (B)	0.25	1.5	82	10	52
6 (CF)	1.5	0.1	69	17	45
7 (CF) <sup>b</sup>	16.5	5.2	52	28	66
8 (CF)	0.9	0.5	37	7	24
9 (B)	0.2	0.3	24	0	16
10 and 11 (B)	0	0	5 <sup>c</sup>	ND <sup>d</sup>	ND
12 and 13 (CF)	0	0	-3 <sup>e</sup>	ND	ND
14 to 18 (B)	0	0	ND	ND	ND

<sup>a</sup> CF, Cystic fibrosis; B, bronchiectasis.

<sup>b</sup> This secretion was obtained directly by aspiration from the large airways.

<sup>c</sup> Mean value derived from sputum sol samples 10 and 11.

<sup>d</sup> ND, Not done.

<sup>e</sup> Mean value derived from sputum sol samples 12 and 13 (CBF in sol samples was more rapid than in control medium).

ciliary slowing on its own. Neither pyocyanin nor 1-hydroxyphenazine was detected in the five sputum sol samples (samples 14 to 18) from patients who had never been colonized by *P. aeruginosa*.

In contrast to our earlier work on culture filtrates (14), in which HPLC-UV profile analysis was sufficient to define and quantify pyocyanin and 1-hydroxyphenazine, the occurrence in sputum of UV light-absorbing impurities which eluted with retention times similar to those of pyocyanin generally necessitated full UV analysis after HPLC for identification and quantification of the test substance. The presence of pyocyanin was only accepted when the UV spectrum (in 0.1 M hydrochloric acid) of the test substance after HPLC was essentially indistinguishable from that of the authentic material, with a  $\lambda_{\max}$  of 278 nm ( $\epsilon_{\max}$ , 50,000) and characteristic multiple absorbances between 350 and 400 nm (14). 1-Hydroxyphenazine from three sol samples (samples 1, 5, and 7) was similarly quantified after HPLC ( $\lambda_{\max}$ , 273 nm;  $\epsilon_{\max}$ , 27,000). In the remaining sol samples, 1-hydroxyphenazine was identified as a UV light-absorbing species because of the concomitant rise and fall of the UV absorbance in both wavelength channels (254 and 280 nm). Measurement of the  $A_{280}/A_{254}$  ratio (which is 0.3 for authentic 1-hydroxyphenazine) gave added confidence in the assignment of 1-hydroxyphenazine in these cases. In three cases in which sufficient material was available, liquid chromatography-thermospray mass spectrometry was applied to define the presence of pyocyanin unequivocally ( $M + H^+ m/z$  211/212) (14). With HPLC retention times, full UV spectra and, in three cases, thermospray mass spectra, confidence in the structural assignments of pyocyanin was high.

Purified pyocyanin extracted from each phenazine-containing sol sample was dissolved in an equivalent volume of PBS. Each sample caused significant ( $P < 0.001$ ) ciliary slowing after 4 h (11 to 100%) when compared with a control containing PBS alone. In three sol samples (samples 1, 5, and 7), the extracted 1-hydroxyphenazine (dissolved in an equivalent volume of PBS) also caused significant ( $P < 0.001$ ) ciliary slowing (24 to 34%). The effect of 1-hydroxyphenazine was immediate and associated with ciliary dyskinesia, while that of pyocyanin was slowly progressive and associated with epithelial disruption (15).

Three of the control sol samples (from patients who had never been colonized by *P. aeruginosa*) clearly contained no

pyocyanin or 1-hydroxyphenazine, since no appreciable UV responses were present in the HPLC profiles at the appropriate retention times. UV responses were observed in the other two controls in the HPLC elution region of pyocyanin and 1-hydroxyphenazine, although the  $A_{280}/A_{254}$  ratios differed from those of authentic standards. The full UV spectra of these substances were obtained in 0.1 M hydrochloric acid. The spectra were not consistent with the presence of pyocyanin or 1-hydroxyphenazine; each spectrum had a  $\lambda_{\max}$  of <273 nm and no absorbance between  $A_{300}$  and  $A_{400}$ .

Bioactivity was always extractable with chloroform (Table 1). Free polymorphonuclear leucocyte elastase in purulent sputum (11) inhibits ciliary beating and damages epithelium (10, 13). Inhibition of all measurable elastase activity in the sol phase significantly reduced the bioactivity in six of nine secretions but, in each case, to a lesser extent than did chloroform extraction. This result suggests that low-molecular-weight, hydrophobic bacterial species were largely responsible for the changes observed in ciliary function. To determine whether other factors, apart from the two phenazines, were present in sputum, we obtained a HPLC profile of biological activity following chloroform extraction of an active sol sample. Three peaks of cilioinhibitory activity were observed: pyocyanin, 1-hydroxyphenazine, and a more polar, as-yet-unidentified, species which eluted before the phenazines in HPLC. A number of 2-alkyl-4-hydroxyquinolines and rhamnolipids from *P. aeruginosa* slow rabbit ciliary beating (6). 2-Heptyl-4-hydroxyquinoline was synthesized from its *N*-oxide (Sigma Chemical Co.) by reduction with titanium trichloride. 2-Heptyl-4-hydroxyquinoline (7 μg/ml) caused 41% ciliary slowing of immediate onset and associated with epithelial disruption. However, both 2-heptyl-4-hydroxyquinoline and rhamnolipid eluted considerably later than did pyocyanin and 1-hydroxyphenazine in HPLC and were not the polar species.

Purulent sputum may contain both bacterial and host products which contribute to tissue damage during chronic bronchial infections (2, 12). In most cases the sol phase of the secretions tested was toxic for human ciliated epithelium. It is possible that other active factors are present in the discarded gel phase. Pyocyanin and 1-hydroxyphenazine contributed a significant proportion of sol phase toxicity. Resolution of each of these compounds from sputum has not previously been achieved (7), although it has long been

suggested that they are responsible for the blue-green coloration of wounds infected by *P. aeruginosa* (5). Being hydrophobic and of low molecular weight, pyocyanin and 1-hydroxyphenazine are of low immunogenicity and may penetrate secretions easily, enabling them to evade neutralization by the host antibody response, in contrast to other pseudomonas virulence factors (11). The levels measured here prove *in vivo* production of phenazines by *P. aeruginosa* in the respiratory tract. If ciliary inhibition were to occur *in vivo* it would perturb the first line of bronchial defense mechanisms. The phenazines and other bacterial components of purulent sputum yet to be characterized may have a pathogenic role during *P. aeruginosa* colonization of and contiguous spread within the bronchial tree.

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