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Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells

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Denning, Gerene M., Shankar S. Iyer, Krzysztof J. Reszka, Yunxia O'Malley, George T. Rasmussen, and Bradley E. Britigan. Phenazine-1-carboxylic acid, a secondary metabolite of *Pseudomonas aeruginosa*, alters expression of immunomodulatory proteins by human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 285: L584–L592, 2003. First published May 23, 2003; 10.1152/ajplung.00086.2003.—*Pseudomonas aeruginosa* is a gram-negative bacterium that causes both acute and chronic lung disease in susceptible patient populations. *P. aeruginosa* secretes numerous proteins and secondary metabolites, many of which have biological effects that likely contribute to disease pathogenesis. An unidentified small-molecular-weight factor was previously reported to increase IL-8 release both in vitro and in vivo. To identify this factor, we subjected the <3-kDa fraction from *P. aeruginosa*-conditioned medium to HPLC analysis. A peak fraction that stimulated IL-8 release was found by mass spectrometry to have a molecular mass (MM) of 224 Da. On the basis of this MM and other biochemical properties, we hypothesized that the factor was phenazine-1-carboxylic acid (PCA). Subsequent studies and comparison with purified PCA confirmed this hypothesis. Purified PCA exhibited a number of biological effects in human airway epithelial cells, including increasing IL-8 release and ICAM-1 expression, as well as decreasing RANTES and monocyte chemoattractant protein-1 (MCP-1) release. PCA also increased intracellular oxidant formation as measured by electron paramagnetic resonance and by an intracellular oxidant-sensitive probe. Antioxidants inhibited PCA-dependent increases in IL-8 and ICAM-1, suggesting that oxidants contributed to these effects. However, in contrast to the related phenazine compound pyocyanin, PCA did not oxidize NAD(P)H at physiologically relevant pH, providing preliminary evidence that PCA and pyocyanin may have distinct redox chemistries within the cell. Thus PCA is a biologically active factor secreted by *P. aeruginosa* that has several activities that could alter the host immune and inflammatory response and thereby contribute to bacterial disease pathogenesis.

inflammation; bacterial pathogenesis; reactive oxygen species

PSEUDOMONAS AERUGINOSA is an opportunistic human pathogen that causes serious and often fatal infections in susceptible patient populations (3, 12, 30). Currently, the mechanisms by which *P. aeruginosa* causes disease in these patients remain poorly understood. Numerous studies indicate that disease pathogenesis is due, at least in part, to factors secreted by the bacterium. Among these studies are those reporting the effects of a small-molecular-weight (SMW), heat-stable factor on expression of the neutrophil chemokine interleukin-8 (IL-8) and on the monocyte/T cell chemokine receptor-activated normal T cell expressed and secreted (RANTES) (10, 22, 24)

Because of our laboratory's interest in *P. aeruginosa*-mediated changes in gene expression by human airway epithelial cells, we designed experiments to identify this factor. Using several approaches, we determined that phenazine-1-carboxylic acid (PCA) was present in bacteria-conditioned medium and that purified PCA increased IL-8 release by human airway epithelial cells. PCA also altered expression of intercellular adhesion molecule-1 (ICAM-1), RANTES, and monocyte chemoattractant protein-1 (MCP-1).

Several bacterial species, including *Pseudomonas* (4, 8), produce phenazine compounds like PCA, and these compounds have been identified as virulence factors in a number of in vivo model systems (7, 19). The phenazines secreted by *P. aeruginosa* are PCA, pyocyanin, 1-hydroxyphenazine (1-HP), and phenazine-1-carboxamide (4). Biosynthesis of the phenazines is upregulated by nutrient depletion, by high cell density, and by conversion of the bacterium to the biofilm form (8, 19).

Whereas PCA has been determined to act as an important antifungal agent in plant systems (29), little is known about its potential effects in human cells. Other phenazine derivatives from *P. aeruginosa* are redox-active compounds that exert many of their effects by altering oxidative metabolism and/or increasing formation of reactive oxygen species (ROS) (14). ROS in turn can alter cell function and gene expression (2, 18, 20). Whether PCA redox cycles in mammalian cells and, if so, what effects might this redox cycling have, have not been determined. Thus we designed additional experiments to determine whether PCA in-

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creases oxidant formation in human airway epithelial cells and whether these oxidants contribute to any of PCA's biological effects.

MATERIALS AND METHODS

Preparation of bacteria-conditioned medium. Wild-type *P. aeruginosa* PA14 was stored as frozen glycerol stocks and as water stocks. Stocks were also maintained by weekly streaking onto tryptic soy broth agar plates. Plates were then incubated overnight at 37°C and were subsequently stored at 4°C. PA14-conditioned media were prepared as previously described (22). In brief, bacteria were inoculated into glycerol-alanine medium (23) (GACM) or high-phosphate medium (6) (HPCM) and grown for 72 h at 37°C with shaking. Bacteria were then removed by centrifugation (20,000 $g \times 30$ min) and filtration of the supernatant through 0.45-μm filters. The <3-kDa fraction was generated with Centriplus filters (Millipore, Bedford, MA). Fractions were stored at 4°C protected from light.

High-pressure liquid chromatography and mass spectrometry. We injected an aliquot of the <3-kDa fraction from bacteria-conditioned medium (50 μl) into a Microsorb-MV (5 μ) (Varian, Walnut Creek, CA) reversed-phase C_{18} column (250 × 4.6 mm) using a precolumn of the same material. The mobile phase was a gradient that was generated using two solvents, 0.05% trifluoroacetate (TFA) in water and 0.05% TFA in acetonitrile (total run time 30 min). A major peak with biological activity eluting at 19–20 min was then subjected to mass spectrometry (MS) for molecular weight determination. Liquid chromatography and MS were performed by the Molecular Analysis Facility (University of Iowa) on a Hewlett Packard Agilent 1100 LC-MSD detection system, a single quadrupole mass spectrometer equipped with an electrospray ionization source, and run in the positive ion mode.

Protocol for enriching the unidentified factor. PA14 HPCM was acidified to pH 2.5 with concentrated HCl. The medium was then extracted into one-fifth volume of chloroform. After phase separation, the chloroform phase was reextracted into four volumes of 0.01 N NaOH. The aqueous phase was adjusted to pH <2, and a second chloroform extraction was performed. The chloroform phase was then dried down under N_2, and the extract was redissolved in 0.01 N NaOH. Purified PCA, generously provided by Dr. Linda Thomashow (Washington State University, Pullman, WA), was solubilized in 0.01 N NaOH and was used for comparison in HPLC studies, as well as for subsequent biological assays.

Airway epithelial cell culture. The human respiratory epithelial cell line A549 (no. 185-CCL, American Type Culture Collection) was cultured as previously described (9) in DMEM/Ham's F-12 (1:1; GIBCO-BRL, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 2 mM glutamine, and 500 U/ml each of penicillin and streptomycin (GIBCO-BRL). *Passages 72–100* were used in all studies. Stock cultures were subcultured by twice weekly trypsinization and seeding at a 1:10 dilution.

Experimental treatments. To test for biological effects by *P. aeruginosa* factors, A549 cells were cultured in 48-well tissue culture plates until confluent. Bacteria-conditioned media or purified factors were then placed on the cells (200 μl/well), and cultures were incubated for 36 h. For studies of RANTES and MCP-1, all experimental media contained 10 ng/ml TNF-α (22). To determine whether oxidants contributed to the increases in IL-8 and ICAM-1, we pretreated cells for 2 h with 30 mM of the thiol antioxidant *N*-acetyl cysteine (NAC). Cultures were then treated with and without the indicated concentration of PCA (0–50 μM) for 36 h in the continued presence of NAC. At the end of the treatment period, the media were harvested for determination of IL-8 release, and the cells were used to assay for ICAM-1 surface expression.

ELISA. We measured cytokines in the medium by ELISA using matched antibodies from R&D Systems (Minneapolis, MN) as previously described (9, 22). Standard curves were in the range of 15–1,000 pg/ml for IL-8 and RANTES and 39–2,500 pg/ml for MCP-1.

Cell-based assay for ICAM-1. Cultures in 48-well plates were washed twice with Dulbecco's PBS (DPBS)/Ca^{2+}/Mg^{2+} containing 0.01% BSA (assay buffer). All subsequent steps were done at 37°C with rocking, all antibodies were diluted in assay buffer (100 μl/well), and all incubations were followed by three washes with assay buffer. Cultures were incubated for 1 h stepwise with assay buffer, with anti-ICAM-1 (anti-CD54, 1:2,000 of a 100 μg/ml stock; Pharmingen, BD Biosciences, San Diego, CA), with biotinylated anti-mouse IgG (1:2,000 of a 500 μg/ml stock, Pharmingen), and with horseradish peroxidase-streptavidin (1:1,000 of 1 mg/ml stock; Pierce, Rockford, IL). Antibody binding was visualized by addition of 3,3',5,5'-tetramethylbenzidine substrate (Sigma, St. Louis, MO), and color development was stopped by addition of 0.5 M H_2SO_4. We read absorbances at 450 nm using a minus primary antibody control as a blank.

Spin trapping and electron paramagnetic resonance. We performed electron paramagnetic resonance (EPR) using A549 cells cultured in 24-well tissue culture plates. In brief, the cells were washed twice with Hanks' balanced salt solution. For measurement of superoxide formation, 1 ml of reaction buffer [DPBS/Ca^{2+}/Mg^{2+}, 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and 0.1 mM diethylenetriaminepentaacetic acid, sodium salt (DTPA)] without (buffer control) or with 50 μM PCA alone or PCA with 100 U/ml superoxide dismutase (SOD) was placed on the cells. Samples were incubated for 30 min at 37°C, and then the solutions were removed from the cells and immediately analyzed by EPR with the Brüker EMX EPR spectrometer operating at *X*-band with 100-kHz modulation. Parallel incubations for each condition were done without A549 cells (no cell controls). To determine whether hydroxyl radical (·OH) was formed, we used a similar protocol, replacing the spin trap DMPO with the spin trap *N*-tert-butyl-α-(4-pyridyl)nitrone *N*′-oxide (POBN, 10 mM) and 1% ethanol. As a positive control, the POBN/ethanol assay buffer was combined with a ·OH-generating system: 10 μM $FeSO_4$ and 10 μM H_2O_2. Spectra represent the average of seven scans with the following instrument settings: microwave power 25.14 mW, modulation amplitude 1 G, time constant 81.9 ms, and sweep time 80 G/41.9 s.

Studies with an oxidant-sensitive fluorescent probe. A549 cells were cultured to confluence in 24-well plates. Stock solutions of 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, 10 mM in DMSO) (Molecular Probes, Eugene, OR) were diluted to 20 μM in DPBS/Ca^{2+}/Mg^{2+} with 0.01% BSA, and 100 μl were placed in each well. Cultures were incubated for 1 h at 37°C. An equal volume of the buffer solution containing a 2× concentration of PCA or pyocyanin was then added to the indicated well. We monitored fluorescence increases (λexcitation/λemission, 485/538) over time at 37°C using the Fluostar Optima microplate fluorometer (BMG Laboratories, Durham, NC). As a control to test whether PCA directly oxidizes CM-DH_2DCFDA, we activated the probe by hydrolyzing it with 10 mM NaOH for 60 min in the dark. The probe was then neutralized by dilution in PBS. Activated probe (10 μM) was

combined with PCA (0–50 μM), and fluorescence was measured (0–24 h) over time at room temperature.

Oxidation of NAD(P)H. To test the pH dependence of NAD(P)H oxidation by the phenazine compounds, DPBS/Ca^{2+}/Mg^{2+} (pH 7.4), phosphate buffer (pH 7.0), or acetate buffer (pH 5.0) was combined in a quartz cuvette with 50 μM pyocyanin or PCA and 250 μM NADH or NADPH. We generated absorbance spectra (200–700 nm) at increasing times (10 s–10 min) following NAD(P)H addition using the Hewlett-Packard Agilent model 8453 spectrophotometer (Agilent Technologies, Chesterfield, MO). To determine the optical absorbance due to NAD(P)H, values at 340 nm were corrected for the contribution made by pyocyanin or PCA.

RESULTS

HPLC analysis of bacteria-conditioned media. To identify components of *P. aeruginosa*-conditioned medium with biological activity, we analyzed the <3-kDa fraction from PA14 bacteria-conditioned medium by HPLC. Representative tracings from this analysis are shown in Fig. 1, *A* and *B*. Fractions from the column were then collected, dried down, resuspended in airway culture medium, and placed on A549 cells. After 36 h, the media were collected and assayed for IL-8 release by ELISA. For GACM (Fig. 1*A*), fractions corresponding to pyocyanin and to those fractions that eluted in the range of 1-HP, as well as an unidentified factor, increased IL-8 release (data not shown). In HPCM, measurable activity was seen only in fractions corresponding to the unidentified factor (Fig. 1*B*). This is consistent with our earlier studies, indicating that concentrations of pyocyanin and of 1-HP, which were previously shown to increase IL-8, are low in HPCM (22).

To characterize the unidentified factor further, we subjected the <3-kDa fraction to HPLC/MS. Figure 1*C* shows the spectrum that was generated. The peak at 225.1 Da represents the unidentified factor ionized by addition of a proton (1 kDa). Subtracting the weight of the proton gives a molecular mass for the factor of 224.1 Da. The peak at 207.1 represents this ionized species with a loss of water, and the shoulders on each peak are due to the presence of the ^{13}C isotope.

Partial purification and identification of PCA. P. aeruginosa secretes a wide variety of pigments and other secondary metabolites (19). From the structure of these metabolites, we calculated that one, PCA (Fig. 2), had a molecular mass of 224 Da. Preliminary identification of the unknown factor as PCA was consistent with both this molecular mass as well as its reported chemical properties (22). On the basis of this initial identification, we sought to purify PCA from bacteria-conditioned medium by acidification and extraction into chloroform (see MATERIALS AND METHODS). The resulting fraction was then reanalyzed by HPLC and compared with purified preparations of PCA and of the other biologically active phenazine derivatives, pyocyanin and 1-HP (Fig. 2).

Using a detection wavelength of 220 nm, we observed two peaks (see Fig. 3: ‡19.6 min and *20.6 min) in the enriched fraction (Fig. 3*A*). Addition of pyocyanin (Py, 9.1 min) and 1-HP (HP, 18.9 min) to this fraction indicates that neither of the two peaks corresponded to these compounds (Fig. 3*B*).

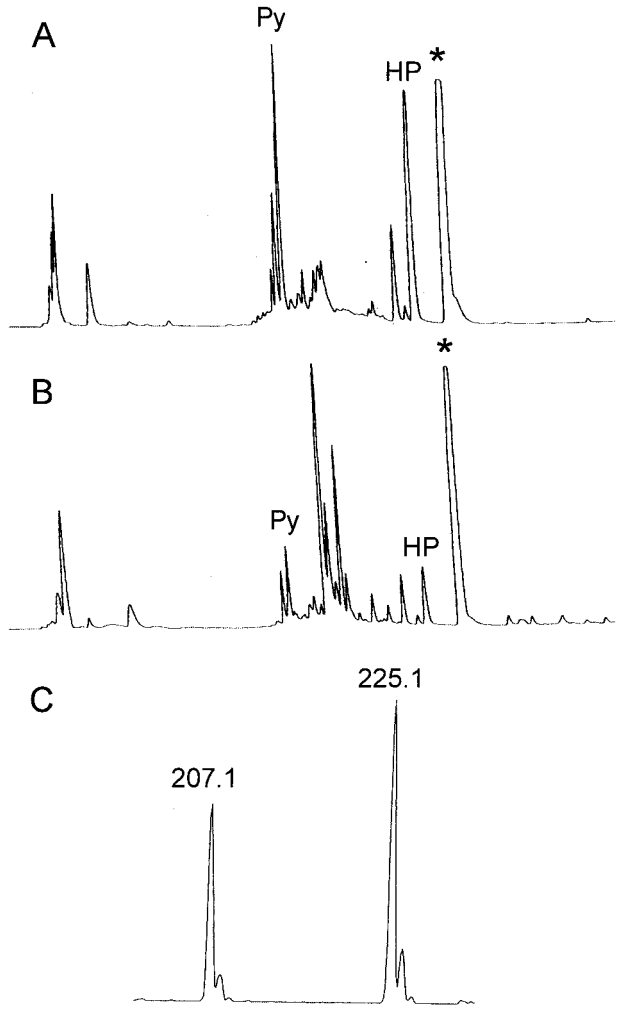

Fig. 1. Analysis of the <3-kDa fraction by HPLC. Representative (n = 4) HPLC elution profiles are shown for the <3-kDa fraction from PA14 glycerol/alanine-conditioned medium (GACM, *A*) and high-phosphate-conditioned medium (HPCM, *B*) using a detection λ of 220 nm. On the basis of the elution profile of purified compounds, the peaks corresponding to pyocyanin (Py, 13.1 min) and 1-hydroxyphenazine (HP, 18.7 min) are indicated. *An unidentified compound with an elution time of 20.2 min. *C*: liquid chromatography-mass spectrometry analysis of the peak corresponding to the unidentified compound. The major peak at 225.1 represents the compound with addition of a proton, and the peak at 207.1 represents the ionized compound with a loss of a water molecule.

In subsequent experiments, when a detection wavelength of 257 nm (a maximal wavelength for PCA) was used, the latter of the two peaks in our enriched fraction was enhanced (Fig. 4*A*, 19.2 min). Note that in these experiments the former peak (‡) had an elution time of ~17.1 min.

To determine further whether the major peak represented PCA, we ran a purified preparation of PCA under identical conditions. The purified PCA had an elution time of 19.3 min (Fig. 4*B*), one nearly identical to the peak in Fig. 4*A*. Because manual injection slightly altered elution profiles, the enriched fraction

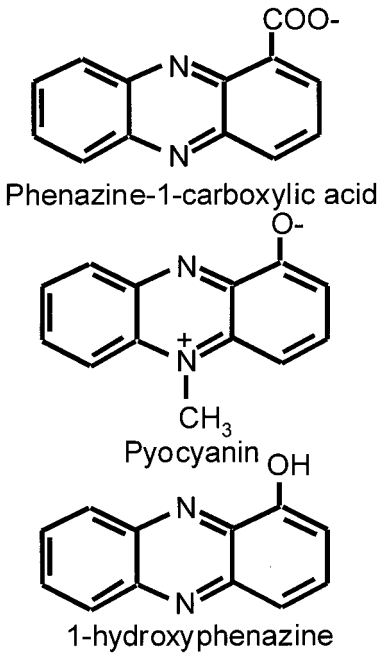

Fig. 2. Structures of phenazine derivatives from *Pseudomonas aeruginosa*. Shown is the predicted structure of the phenazine derivatives at pH 7.4.

was combined with the purified PCA, and the mixture was reanalyzed. Under these conditions, a single peak was observed (Fig. 4*C*, 19.0 min). These data indicate that our purification protocol enriched for two compounds, one of which was PCA. Identification of the other compound is currently underway.

Biological effects of purified PCA on human airway epithelial cells. To determine whether PCA has biological effects on airway epithelial cells, we exposed A549 cells to increasing concentrations of purified PCA or our enriched preparation for 36 h. The media were then harvested for measurement of cytokine release, and the cells were assayed for ICAM-1 surface expression. Results were compared with earlier studies using the <3-kDa fraction from PA14 HPCM (Table 1). As was observed with the <3-kDa fraction, both purified PCA and our enriched preparation increased IL-8 and ICAM-1 expression. Also similar to the <3-kDa fraction, both preparations decreased TNF-dependent RANTES and MCP-1 release. All observed effects were statistically significant ($P < 0.01$).

Note that caution must be exercised in attempting to compare the results obtained with each of the three preparations. All three (<3-kDa fraction, enriched preparation, and purified PCA) were tested in separate experiments, and some variability in both the basal levels of protein expression as well as the magnitude of changes was observed from experiment to experiment. Also, we do not as yet know whether the other compound present in our enriched preparation (Fig. 3*A*) has biological activity either alone or in combination with PCA. And finally, while PCA is a major component of the <3-kDa fraction of PA14 HPCM (Fig. 1), other factors with similar biological activity may also be present in this crude preparation. Moreover, both our enriched fraction and the SMW fraction were studied as a percentage of the medium added to the wells, and, therefore, the concentration of PCA in those samples was not known.

EPR studies. Pyocyanin is thought to exert many of its biological effects, including increasing IL-8 (9), by increasing oxidant formation within the cell (11). Because PCA is structurally similar to pyocyanin, we initially hypothesized that PCA may exert prooxidant effects in mammalian cells. Thus it was of interest to determine whether PCA, like pyocyanin (9, 11, 28), stimulates intracellular oxidant formation in human airway epithelial cells.

As one approach to measure oxidant formation, we used the spin trap DMPO, a compound that reacts with superoxide anion ($O_2^-\cdot$) or with ·OH to form the DMPO/·OH adduct. This adduct exhibits a characteristic spectrum when analyzed by EPR (Fig. 5*A*) (splitting constants for nitrogen and hydrogen, respectively: a_N = 14.9 G, a_H^β = 14.9).

In the absence of cells, 50 μM PCA did not increase the EPR signal above buffer control (not shown), indicating that there was no generation of oxidants without cells. Conversely, when A549 cells were exposed to PCA for 30 min, there was a significant increase in the

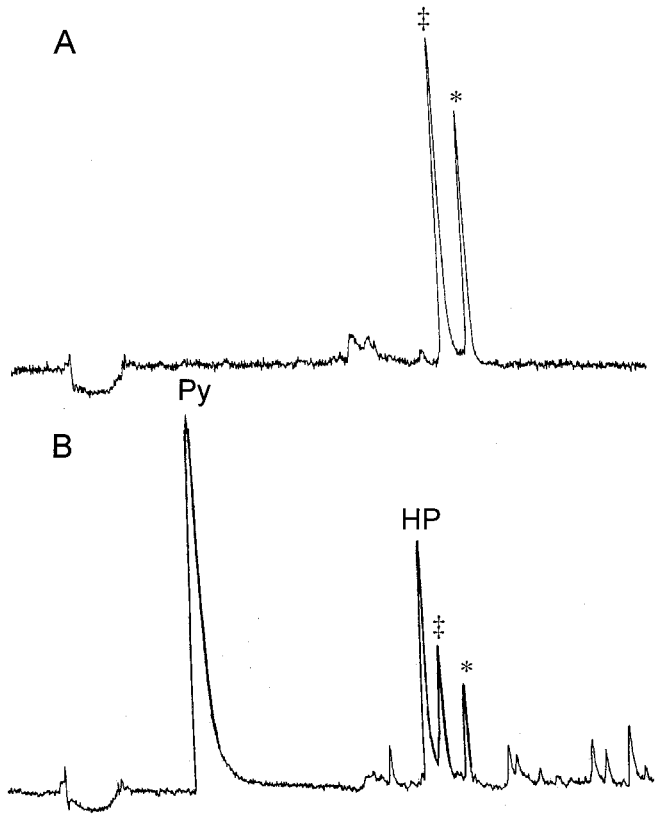

Fig. 3. HPLC analysis of an enriched fraction from PA14-conditioned medium. The <3-kDa fraction from PA14 HPCM was subjected to a purification protocol as described in MATERIALS AND METHODS. HPLC tracings are shown for the enriched fraction alone (*A*) and for the enriched fraction combined with purified pyocyanin and 1-HP (*B*). Detection λ, 220 nm; ‡19.6 min and *20.6 min.

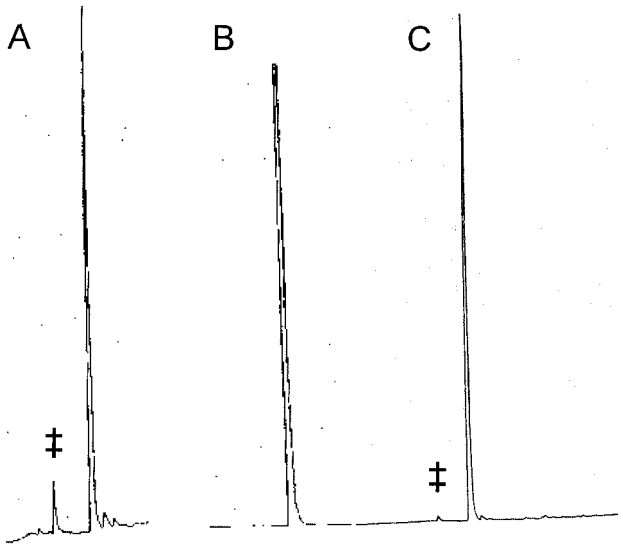

Fig. 4. Comparison of the enriched fraction with purified phenazine-1-carboxylic acid (PCA). The <3-kDa fraction from PA14 HPCM was subjected to a purification protocol as described in MATERIALS AND METHODS. An aliquot of the enriched preparation (*A*), purified PCA (*B*), or a mixture of the two (*C*) was then analyzed by HPLC. Figure shows tracings using one of the maximal detection wavelengths for PCA (257 nm). ‡Unidentified peak also shown in Fig. 3.

EPR signal attributable to DMPO/·OH relative to the A549 cell buffer control (Fig. 5*A*). The signal intensity was significantly decreased if SOD was present during the incubation period (Fig. 5*A*). SOD-dependent inhibition was 78 ± 6% (means ± SE, $n = 4$) with a range of 65–85%. These data confirm that the formation of DMPO/·OH was mediated, at least in part, by O_2^-· and thus that PCA stimulated O_2^-· formation by A549 cells. In these experiments, we measured DMPO/·OH in the extracellular medium, and its formation was decreased by extracellular SOD. These observations suggest that oxidants were formed at or near the plasma membrane but do not rule out the possibility that oxidants were also produced at locations deeper within the cell.

To determine whether OH· was formed under these conditions, we used a spin trapping system (POBN/1% ethanol) that detects OH· but not O_2^-·. With this system, production of OH· leads to formation of the spin adduct POBN/·CH_2OHCH_3 with the characteristic signal of the POBN carbon-centered radical ($a_N = 15.7$ G, $a_H = 2.57$) shown in Fig. 5*B* (Fe^{2+}/H_2O_2). Using this approach, we observed only a very small increase in signal in PCA-treated cells relative to control cells (Fig. 5*B*), suggesting that little or no OH· was formed. A similar absence of significant ·OH formation was observed in endothelial cells exposed to pyocyanin alone (1).

Oxidant-sensitive probe studies. As a second approach to test for oxidant formation, studies were done using the oxidant-sensitive probe CM-H_2DCFDA. The membrane-permeable form of the probe crosses the plasma membrane, where it is converted to a charged form by cellular esterases and is then retained within the cell. Because pyocyanin directly oxidizes this probe (B. E. Britigan, unpublished observation), control studies were first performed to determine whether the probe was directly oxidized by PCA. For these studies, purified PCA (0–50 μM) was combined with activated probe in the absence of cells, and fluorescence was measured at increasing times up to 24 h. Over this time period, there was no increase in fluorescence in PCA-containing samples compared with probe alone (data not shown), suggesting that PCA does not directly oxidize CM-H_2DCFDA.

We next loaded A549 cells with the probe, treated them with and without increasing concentrations of purified PCA for increasing times, and measured fluorescence changes. Figure 6 indicates that PCA treatment increased fluorescence in a time- and concentration-dependent manner, providing further evidence for increased oxidant formation within the cells.

To examine the dose response for this effect, we plotted the increase in fluorescence over the initial 4 h of exposure against the concentration of PCA (Fig. 6, *inset*). The resulting dose-response curve exhibited a plateau. Plateauing of the dose-response curve indicates several possibilities.

First, the plateau might reflect absorption of the excitation wavelength light (485 nm) by PCA during the fluorescence measurements. In that case, increasing concentrations of PCA would decrease the intensity of the light impinging on the fluorescent probe and thereby decrease the fluorescence intensity. To test this possibility, we obtained absorption spectra for increasing concentrations of PCA at pH 7.0. Even at concentrations of PCA as high as 500 μM (absorbance at 365 nm = 1.9 optical density units), the absorbance value at 485 nm was <0.01. These data indicate that PCA does not absorb at the wavelength used for excitation in the fluorescence studies.

A second possibility is that the intracellular probe concentration was limiting and thus the plateau indicates complete oxidation of the probe at higher concen-

Table 1. *Effects of SMW factors from Pseudomonas aeruginosa on human airway epithelial cells*

	ICAM-1, OD units	IL-8, ng/ml	RANTES, ng/ml	MCP-1, ng/ml
Control	0.14 ± 0.02	0.85 ± 0.12	1.8 ± 0.06	17.0 ± 2.0
<3 kDa	0.25 ± 0.01	4.0 ± 0.27	0.2 ± 0.05	6.0 ± 2.0
Control	0.06 ± 0.01	0.80 ± 0.14	2.6 ± 0.43	33.0 ± 9.0
PCA	0.38 ± 0.09	5.4 ± 1.72	0.2 ± 0.06	10.5 ± 0.36
Control	0.09 ± 0.01	0.95 ± 0.056	3.3 ± 0.62	27.4 ± 2.2
PCA std	0.37 ± 0.02	3.5 ± 0.92	1.6 ± 0.63	19.4 ± 0.67

Data represent the average ± SD of triplicate samples. A549 cells were exposed for 36 h to 20% of the <3-kDa fraction from PA14 high-phosphate-conditioned medium (<3 kDa), 5% of a fraction enriched for phenazine-1-carboxylic acid (PCA), or 25 μM of a purified PCA standard (PCA std). Samples to be measured for the effect of bacterial factors on receptor-activated normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein (MCP-1) were also treated with 10 ng/ml TNF-α. The medium was then harvested, and cytokines in the medium were measured by ELISA. ICAM-1 surface expression was measured by a cell-based ELISA method. $P < 0.01$ for each set of conditions. Similar results were seen in 3 or more independent experiments. OD, optical density; SMW, small molecular weight.

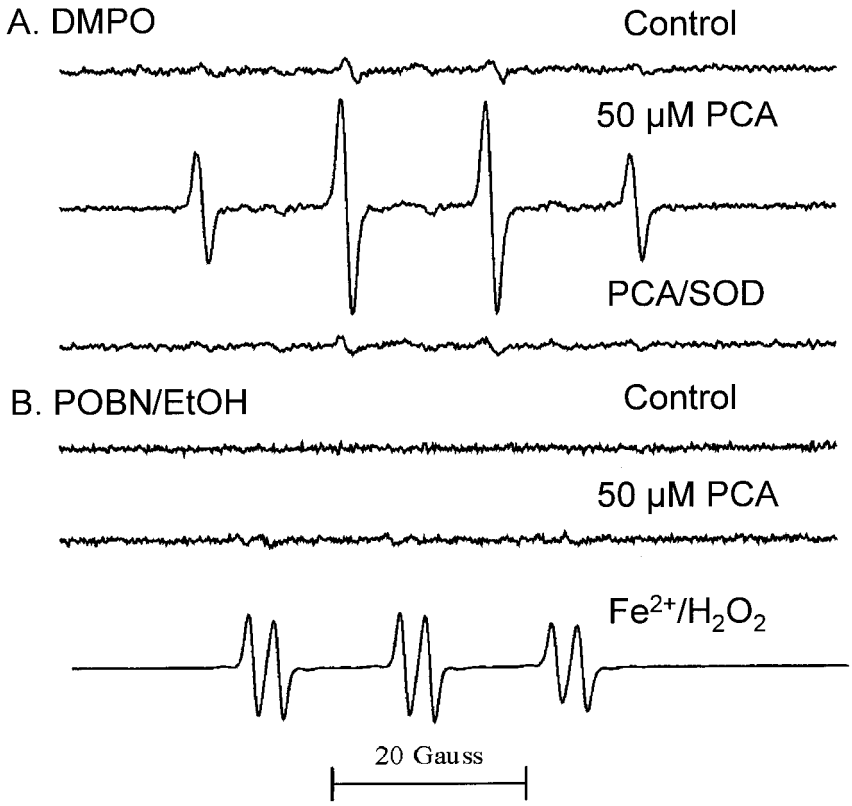

Fig. 5. Electron paramagnetic resonance (EPR) studies using the spin traps 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and *N*-tert-butyl-α-(4-pyridyl)nitrone *N*′-oxide (POBN). *A*: confluent cultures of A549 cells were exposed for 30 min at 37°C to DMPO reaction buffer [DPBS/Ca^{2+}/Mg^{2+}, 100 mM DMPO, and 0.1 mM diethylenetriaminepentaacetic acid, sodium salt (DTPA)] alone (Control) or to reaction buffer with PCA alone (50 μM PCA) or 50 μM PCA combined with 100 U/ml superoxide dismutase (SOD) (PCA/SOD). *B*: cultures of A549 cells were similarly treated with POBN reaction buffer (DPBS/Ca^{2+}/Mg^{2+}, 10 mM POBN, 1% ethanol, and 0.1 mM DTPA) alone (Control) or reaction buffer with PCA (50 μM PCA). As a positive control, POBN reaction buffer was combined with 10 μM $FeSO_4$ and 10 μM H_2O_2 (Fe^{+2}/H_2O_2). EPR measurements were then performed on the samples as described in MATERIALS AND METHODS (n = 3). The receiver gains for the spectra shown were as follows: all spectra in *A*, 2×10^6; control and PCA spectra in *B*, 5×10^5; Fe^{2+}/H_2O_2 spectrum in *B*, 5×10^4.

trations of PCA. However, we observed that pyocyanin-dependent (50 μM) increases in fluorescence done in parallel cultures of A549 cells were approximately twice those of the highest concentration of PCA tested (100 μM). Representative values for the increase in fluorescence units at 4 h of incubation time were 5,060 ± 990 and 10,250 ± 2,300 (means ± SE, n = 3) for 100 μM PCA and for 50 μM pyocyanin, respectively. Note that these data cannot be used to compare relative oxidant formation by pyocyanin and PCA because, as stated above, pyocyanin directly oxidizes the fluorescent probe. However, the data do provide evidence that the plateau does not reflect complete oxidation of the intracellular probe.

Thus the plateau likely reflects a "saturable" mechanism for PCA-dependent oxidant formation. The identity of this mechanism and the location of oxidant formation are important areas for future study.

Oxidation of NAD(P)H. Pyocyanin can generate superoxide by direct oxidation of reducing sources such as NADH and NADPH (15). To determine whether PCA-dependent oxidant formation within cells might result from reaction with these reducing sources, we used a cell-free system to measure oxidation of NADH or NADPH spectrophotometrically using an absorption wavelength of 340 nm.

Figure 7*A* demonstrates that pyocyanin oxidized NADH at a physiologically relevant pH (7.0) and that this oxidation was enhanced at pH 5. The rate of pyocyanin-dependent oxidation of NADPH was greater than that of NADH, and no enhancement was observed at lower pH (Fig. 7*B*). In contrast to pyocyanin, PCA did not appreciably oxidize NADH (Fig. 7*A*) or NADPH (Fig. 7*B*) at pH 7. Moreover, oxidation of both NADH and NADPH by PCA at pH 5 was considerably slower than oxidation of NADH by pyocyanin under any of the conditions studied.

Effect of antioxidants. Our studies show that purified PCA increases oxidant formation within airway epithe-

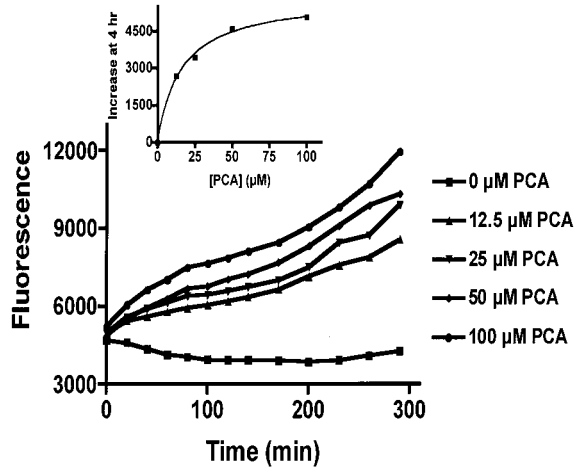

Fig. 6. Intracellular oxidant formation as measured with an oxidant-sensitive fluorescent probe. A549 cells were loaded with 5- (and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DH_2DCFDA, 20 μM) for 1 h at 37°C. Increasing concentrations of PCA (final 12.5–100 μM) were then added to the wells, and fluorescence changes (λexcitation/λemission, 485/538 nm) at 37°C were measured over time. Values represent the average of triplicate samples. *Inset*: graph of increase in fluorescence at 4 h vs. PCA concentration. Similar results were seen in 3 independent experiments.

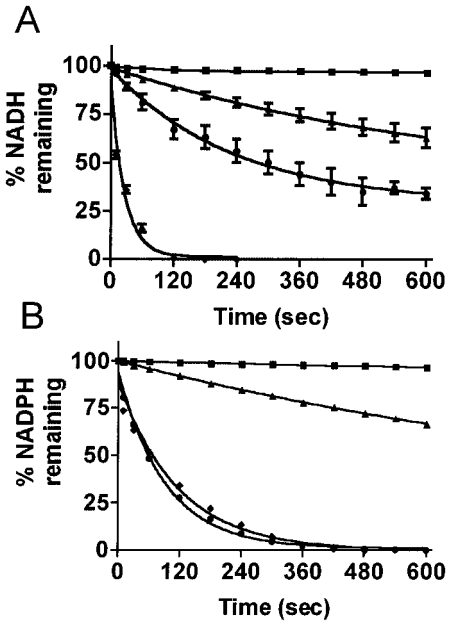

Fig. 7. Oxidation of NAD(P)H. Buffer (pH 7 or 5) containing 50 μM pyocyanin or PCA was combined in a quartz cuvette with 250 μM NADH (*A*) or NADPH (*B*). The absorbance spectrum (200–700 nm) was then measured at increasing times (10 s–10 min): PCA, pH 7.0, ■; PCA, pH 5.0, ▲; pyocyanin, pH 7.0, ●; pyocyanin, pH 5.0, ◆. The absorbance at 340 nm due to NAD(P)H at each time point was determined by subtracting the contribution of pyocyanin or PCA. Values represent the means ± SE (n = 4).

lial cells and alters expression of immunomodulatory proteins. To determine whether increased protein expression (IL-8 and ICAM-1) is due, at least in part, to PCA-dependent oxidant formation, we tested the effect of the antioxidant NAC. At 30 mM, NAC inhibited PCA-dependent increases in both IL-8 (Fig. 8*A*) and ICAM-1 (Fig. 8*B*): $P < 0.001$ for each concentration of PCA tested. In previous studies, we found that NAC also inhibited increases in IL-8 and ICAM-1 in response to the <3-kDa fraction of bacteria-conditioned medium (data not shown). These results suggest that oxidants contribute to PCA-dependent increases in IL-8 and ICAM-1.

DISCUSSION

Previous studies demonstrate that SMW factors from *P. aeruginosa* increase IL-8 release in vitro (10, 22, 24) as well as IL-8 release and neutrophil influx in vivo (16). Two of the factors that increase IL-8, pyocyanin, and 1-HP have been purified, and their biological effects have been studied. However, the SMW, heat-stable fraction from bacteria-conditioned medium increases IL-8 even under conditions where there is negligible production of pyocyanin and 1-HP (22). We showed that this fraction, in addition to increasing IL-8, inhibits RANTES release by TNF- and IL-1-treated A549 cells and provided evidence that the factor(s) involved are not LPS or autoinducer (22).

In the current report, we demonstrate that PCA is a component of the SMW fraction from bacteria-conditioned medium and that purified PCA has multiple biological activities in human airway epithelial cells that are similar to the biological activities of the <3-kDa fraction of bacteria-conditioned medium. Moreover, PCA increased oxidant formation within airway cells, and antioxidants inhibited PCA-dependent increases in IL-8 and ICAM-1, suggesting that oxidant formation contributed to these effects. To our knowledge, this is the first report that PCA has effects in human cells, including stimulating oxidant formation. Of note, although PCA is a major component of the <3-kDa fraction (Fig. 1), we cannot rule out a contribution by factors, in addition to pyocyanin, 1-HP, and PCA, to the biological activity of this fraction.

We have performed studies directly comparing PCA and pyocyanin. In vitro, we found that pyocyanin-dependent increases in IL-8 release were approximately two- to fivefold higher than PCA-dependent increases over a similar concentration range (0–50 μM). Interestingly, in preliminary studies using a mouse airway model, equal concentrations of pyocyanin and PCA (50 μM) stimulated a nearly identical influx of mouse neutrophils. These data indicate that quantitative differences observed in vitro may not accurately predict the relative potency of pyocyanin and PCA in vivo.

Additionally, both pyocyanin and the <3-kDa fraction of bacteria-conditioned medium have similar effects on IL-8, ICAM-1, and RANTES in both cell lines and in polarized monolayers of primary airway epithe-

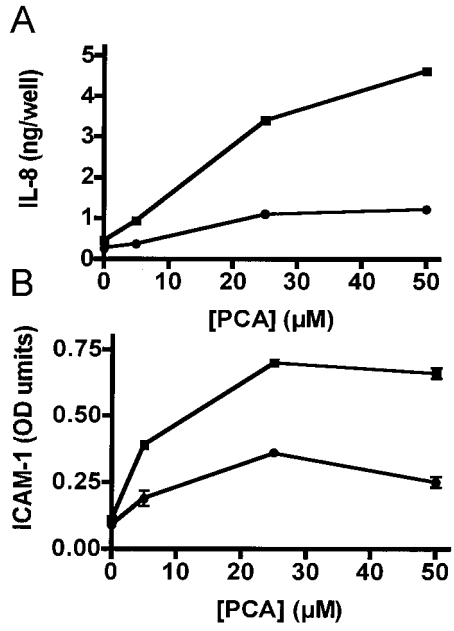

Fig. 8. Effect of *N*-acetyl cysteine (NAC) on PCA-dependent increases in IL-8 and ICAM-1. A549 cells were pretreated without (■) and with 30 mM NAC (●) and then exposed for 36 h to the indicated concentration of PCA in the continued presence of NAC. Media were assayed for IL-8, and cells were assayed for ICAM-1 as described in MATERIALS AND METHODS. Values represent the means ± SE (n = 6). Similar results were seen in 2 independent experiments. *A* and *B*: using Student's t-test, we observed statistically significant differences, $P < 0.001$ (with vs. without NAC), for each concentration of PCA tested.

lial cells (data not shown). Similar to these studies, it will be important to expand on our reported findings with PCA using model systems in addition to A549 cells.

The mechanism for PCA-dependent oxidant formation is currently unknown. At neutral pH, PCA is negatively charged (Fig. 2), making it less electrophilic (able to accept electrons) than pyocyanin, which is a zwitterion at pH 7. This is consistent with the observation that pyocyanin, but not PCA, oxidizes NAD(P)H at pH 7. At pH 5, some of the PCA molecules may be protonated and hence may be more electrophilic. This is consistent with our observation that PCA oxidizes NAD(P)H, albeit slowly, at pH 5: pyocyanin is positively charged at pH 5 and thus is more electrophilic. Whereas our data suggest that NAD(P)H may not directly reduce PCA within the cell, we cannot rule out the possibility that the intracellular environment may facilitate direct reduction. Alternatively, the reduction of PCA within the cell may require enzymatic activity by diaphorases or other reductases that could promote NAD(P)H reduction of PCA, as was observed with the redox-active compound paraquat (15). Finally, PCA itself may not directly generate oxidants but rather may be metabolized by airway cells to other compounds that are redox active (e.g., 1-hydroxyphenazine).

Several bacterial species produce one or more of the 50 known phenazine compounds. These species include *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (8). Whereas PCA is made by several species, pyocyanin and 1-HP are unique to *P. aeruginosa* (5). Biosynthesis of PCA in *Pseudomonas* is controlled by two seven-gene operons, *phz*A1B1C1D1E1F1G1 and *phz*A2B2C2D2E2F2G2 (25). PCA is formed by condensation of two molecules of chorismic acid with a simultaneous decarboxylation at the 6 position (26). Pyocyanin is formed from PCA by the stepwise action of a phenazine-specific methyltransferase (*phz*M), forming 5-methyl-1-carboxylic acid betaine followed by a flavin-containing monooxygenase (*phz*S) that converts the carboxylic acid group at *position 1* to a hydroxyl group (25). The same flavin-containing monooxygenase (*phz*S) directly converts PCA to 1-HP (25).

Production of the phenazines is low in log phase growth. This is due to catabolite repression by carbon and nitrogen sources. Increased production in late log and stationary phase results both from nutrient depletion as well as from activation of the quorum-sensing systems *rhl*I/R and *las*I/R (8).

Our findings are of considerable significance for the following reasons. First, PCA is a precursor for pyocyanin and for 1-HP (19). Because pyocyanin is found in sputum and bronchoalveolar lavage fluid from patients with *P. aeruginosa*-associated lung disease (31), it seems highly likely that PCA is present in the airway of some patients. Future studies will be done to test this important hypothesis.

Second, synergy is observed between factors in increasing IL-8. Consistent with this observation are our findings that bacteria-conditioned medium containing both pyocyanin and other SMW factors, including PCA, was considerably more potent than conditioned medium without pyocyanin (22). This suggests that the presence of more than one of the phenazine compounds could lead to more marked effects on the host immune response.

Third, phenazine derivatives have been shown to be virulence factors in plant, animal, and human systems (7, 19). Increased expression of the proinflammatory mediators IL-8 and ICAM-1 in response to PCA could have important physiological implications in the infected host. For *P. aeruginosa*-associated airway disease, influx and activation of neutrophils with excessive release of ROS and neutrophil elastase are thought to contribute significantly to the airway damage that occurs (13, 27). Moreover, decreased expression of monocyte and T cell chemokines such as RANTES and MCP-1 could reduce influx of these cells and interfere with their immune and anti-inflammatory functions.

Finally, whereas both pyocyanin and 1-HP have been studied for their effects in mammalian model systems (17, 21), PCA has not previously been recognized as having potential relevance to human disease. Moreover, because the physical structure and hence chemical properties of pyocyanin, 1-HP, and PCA are different, understanding the contribution of phenazine derivatives to the pathophysiology of *P. aeruginosa*-associated disease will require not only an understanding of their individual effects, but also an understanding of their combined effects, as well as their effects in the presence of other virulence factors.

Our data indicate that PCA may play a previously unidentified role in *P. aeruginosa*-associated disease. Thus it is of considerable importance that the potential effects of this compound on the host immune and inflammatory responses be further explored.

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REFERENCES

1. **Britigan B, Roeder T, Rasmussen G, Shasby D, McCormick M, and Cox C.** Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. Implications for *Pseudomonas*-associated tissue injury. *J Clin Invest* 90: 2187–2196, 1992.
2. **Brown R and Kelly F.** Role of free radicals in the pathogenesis of cystic fibrosis. *Thorax* 49: 738–742, 1994.
3. **Buret A and Cripps AW.** The immunoevasive activities of *Pseudomonas aeruginosa*. Relevance for cystic fibrosis. *Am Rev Respir Dis* 148: 793–805, 1993.

4. **Byng G, Eustice D, and Jensen R.** Biosynthesis of phenazine pigments in mutant and wild-type cultures of *Pseudomonas aeruginosa*. *J Bacteriol* 138: 846, 1979.
5. **Chang P and Blackwood A.** Simultaneous biosynthesis of pyocyanine, phenazine-1-carboxylic acid, and oxychlororaphine from labelled substrates by *Pseudomonas aeruginosa* Mac 436. *Can J Biochem* 46: 925–929, 1968.
6. **Cox C.** Role of pyocyanin in the acquisition of iron from transferrin. *Infect Immun* 52: 263–270, 1986.
7. **Darby C, Cosma C, Thomas J, and Manoil C.** Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96: 15202–15207, 1999.
8. **Delaney S, Mavrodi D, Bonsall R, and Thomashow L.** *phz0*, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30–84. *J Bacteriol* 183: 318–327, 2001.
9. **Denning G, Wollenweber L, Railsback M, Cox C, Stoll L, and Britigan B.** *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect Immun* 66: 5777–5784, 1998.
10. **DiMango E, Zar H, Bryan R, and Prince A.** Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J Clin Invest* 96: 2204–2210, 1995.
11. **Gardner PR.** Superoxide production by *Mycobacterial* and *Pseudomonas* quinoid pigments phthiocol and pyocyanine in human lung. *Arch Biochem Biophys* 333: 267–274, 1996.
12. **Govan J and Deretic V.** Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 60: 539–574, 1996.
13. **Hanley ME and Repine JE.** Elastase and oxygen radicals: synergistic interactions. *Agents Actions Suppl* 42: 39–47, 1993.
14. **Hassan HM and Fridovich I.** Intracellular production of superoxide radical and of peroxide by redox active compounds. *Arch Biochem Biophys* 196: 385–395, 1979.
15. **Hassan HM and Fridovich I.** Mechanism of the antibiotic action of pyocyanine. *J Bacteriol* 141: 156–163, 1980.
16. **Inoue H, Hara M, Massion P, Grattan K, Lausier J, Chan B, Kaneko T, Isono K, Jorens P, Ueki I, and Nadel J.** Role of recruited neutrophils in interleukin-8 production in dog trachea after stimulation with *Pseudomonas* in vivo. *Am J Respir Cell Mol Biol* 13: 570–577, 1995.
17. **Jackowski JT, Szepfalusi Z, Wanner DA, Seybold Z, Sielczak MW, Lauredo IT, Adams T, Abraham WM, and Wanner PA.** Effects of *P. aeruginosa*-derived bacterial products on tracheal ciliary function: role of O_2 radicals. *Am J Physiol Lung Cell Mol Physiol* 260: L61–L67, 1991.
18. **Kehrer JP.** Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol* 23: 21–48, 1993.
19. **Kerr J.** Phenazine pigments: antibiotics and virulence factors. *Infect Dis Rev* 2: 184–194, 2000.
20. **Kinnula V, Crapo J, and Raivio K.** Biology of disease: generation and disposal of reactive oxygen metabolites in the lung. *Lab Invest* 73: 3–19, 1995.
21. **Lauredo I, Sabater J, Ahmed A, Botvinnikova Y, and Abraham W.** Mechanism of pyocyanin- and 1-hydroxyphenazine-induced lung neutrophilia in sheep airways. *J Appl Physiol* 85: 2298–2304, 1998.
22. **Leidal K, Munson K, and Denning G.** Small molecular weight secretory factors from *Pseudomonas aeruginosa* have opposite effects on IL-8 and RANTES expression by human airway epithelial cells. *Am J Respir Cell Mol Biol* 25: 186–195, 2001.
23. **MacDonald JC.** Biosynthesis of pyocyanine. *Can J Microbiol* 9: 809–819, 1963.
24. **Massion PP, Inoue H, Richman-Eisenstat J, Grunberger D, Jorens PG, Housset B, Pittet JF, Wiener-Kronish JP, and Nadel JA.** Novel *Pseudomonas* product stimulates interleukin-8 release by airway epithelial cells in vitro. *J Clin Invest* 93: 26–32, 1994.
25. **Mavrodi D, Bonsall R, Delaney S, Soule M, Phillips G, and Thomashow L.** Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 183: 6454–6465, 2001.
26. **McDonald M, Mavrodi D, Thomashow L, and Floss H.** Phenazine biosynthesis in *Pseudomonas fluorescens*: branchpoint from the primary shikimate biosynthetic pathway and role of phenazine-1,6-dicarboxylic acid. *J Am Chem Soc* 123: 9459–9460, 2001.
27. **Miller RA and Britigan BE.** The formation and biologic significance of phagocyte-derived oxidants. *J Investig Med* 43: 39–49, 1995.
28. **O'Malley Y, Abdalla M, McCormick M, Reszka K, Denning G, and Britigan B.** Localization of *Pseudomonas* pyocyanin cytotoxicity in human lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 284: L420–L430, 2003.
29. **Thomashow L, Weller D, Bonsall R, and Pierson L III.** Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Appl Environ Microbiol* 56: 908–912, 1990.
30. **Wilson R, Dowling R, and Jackson A.** The effects of bacterial products on airway cells and their function. *Am J Respir Crit Care Med* 154, *Suppl*: S197–S201, 1996.
31. **Wilson R, Sykes DA, Watson D, Rutman A, and Taylor GW.** Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium. *Infect Immun* 56: 2515–2517, 1988.