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Research Letter

Antimicrobial activity of *Pseudomonas* secondary metabolites

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ABSTRACT: **Introduction:** Phenazines are nitrogen-containing heterocyclic molecules produced by fluorescent *Pseudomonas*. These compounds have broad-spectrum antibiotic properties including antimicrobial activity against phytopathogenic fungi and pathogenic bacteria. Several mechanisms of action have been proposed, including inhibition of DNA replication, uncoupling of electron, transport and energy production, and disruption of normal membrane functions resulting in the generation of toxic intracellular oxygen species. **Methods:** Production of antimicrobial metabolites *in vitro* was assayed in NBY supplemented with glucose to a final concentration of 2%. **Results:** Extracts of supernatants from liquid cultures completely inhibited phytopathogenic fungi when incorporated into agar culture at a rateof 20 and 40 µg/ml, this crude extracts was active against all tested fungi. In a disc assay, extracts equivalent to 20 µg gave zones of inhibition of 15 mm and 25 mm for the Gram positive bacteria *Bacillus subtilis* and *Paracoccus paratrophus*, respectively with disc diffusion technique. The purified extracts were analyzed by UV spectroscopy and GC/MS.

KEYWORDS: Antimicrobial activity, phenazines, secondary metabolites

INTRODUCTION

Phenazines (PZs) are well known pigmented, nitrogen-containing heterocyclic secondary metabolites produced by a variety of bacteria, including Pseudomonas, Streptomyces, Nocardia, Sorangium, Brevibacterium, Burkholderia, Methanosarcina and Erwinia.[1–5] Bacteria are the only known source of natural phenazines.[6] The antibiotic properties of these compounds have been known for over 150 years. More than 6,000 PZ containing compounds and more than 100 natural derivatives of PZs have been described. Natural PZs produced by Eubacteria are produced during late growth stages and usually are excreted out of bacterial cells by unknown mechanisms, often at high level. PZs have been studied longer than many other bacterial secondary metabolites.[5] The majority of the work on PZs has focused on the chemical or biological synthesis.[7] The continuing interest in phenazines is due largely to their physiochemical properties, including their oxidation–reduction (redox) properties and their bright pigmentation and ability to change color with pH and redox state.[6] PZs have toxic effects on a broad variety of organisms.[5] One hypothesis is that toxicity results from the generation of reactive oxygen species (ROS).[3] PZs differ in their physical structures based on the various substituents (such as -COOH, -OH, -CH3) added at different positions to the basic heterocyclic ring, and thus may differ in their antibiotic activity. Among various PZs, 2-OH-PCA from *P. chlororaphis* strain 30–84 and Phenazine-1-carboxamide (PCN) from *P. aeruginosa* have greater antifungal activity *in vitro* than Phenazine-1-carboxylic acid (PCA).[5] This might be due to the fact that PCN remains largely protonated at alkaline pH, whereas PCA does not.[7]

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Phenazines continue to be used for many diverse applications, including as electron acceptors and donors, as components of fuel cells, as environmental sensors and biosensors,[6] as central components of antitumor compounds,[6,8] as biological control agents,[7] antimalaria, and antiparasitic activities.[8] The aim of this work was to screen the antimicrobial activity of a phenazine produced by a bacterium isolated in Sétif (Algeria).

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MATERIALS AND METHODS

Origin of the bacterial isolates

Pseudomonas chlororaphis subsp. *aureofaciens* DSM 6698 producing phenazine was obtained as described by Mezaache-Aichour *et al*.[9] These bacteria were maintained on Pseudomonas-specific medium (King's B agar supplemented with 10% glycerol) and stored at 4 °C. *Pseudomonas chlororaphis* subsp. *aureofaciens* strain 30–84 was gifted by Pr HAAS (Lausanne, Switzerland).

Bacterial strains tested

The strains used for antibacterial tests were *E. coli* MC 4100 NCTC 9002, *Bacillus subtilis*, *Pseudomonas diminutus* NCTC 8545, *Paracoccus paratrophus* and *Micrococcus luteus*, which were obtained from Dr. J. Nicklin, School of Biological and Chemical Sciences, Birkbeck College, University of London, UK. While *Salmonella enteridis* was obtained from The LMA of UFA Sétif.

Detection, isolation and purification of phenazines

For the production of the antibiotic, the strain *Pseudomonas chlororaphis* subsp. *aureofaciens* DSM 6698, which produced typically orange colonies (specific color of pyomelanins) on nutrient broth yeast extract (NBY) agar, was grown in NBY liquid medium[10] supplemented with glucose to a final concentration of 2%. The cultures were incubated for 4 to 5 days on a rotary shaker (250 rpm; throw 19 mm) at 28 °C. The antibiotic was extracted twice with an equal volume of ethyl-acetate, after acidification of the cultures to pH 2.0 with concentrated HCl,[11] and then shaking for 1 to 2 h. The resultant emulsion was pooled and then passed through a 0.45 μm membrane to remove bacterial cells. After passing through a short column of anhydrous sodium sulfate to remove water,[10] the ethyl-acetate fraction was evaporated to dryness on a rotary film evaporator at 55 °C.[12] The crude extract was suspended in methanol[13] and chromatographed on silica gel TLC plates (Schleicher and Schull GmbH, Germany) with benzene/acetic acid (95:5) as mobile phase. Chromatograms were examined at 254 nm as described by Thomashow *et al*.[12] Purified extracts were analyzed by UV spectroscopy and subjected to bioassay to determine antimicrobial activity.[10,14] PCA gifted by Dr Thomashow (USDA, University of Washington, Pullman, USA) was used as a standard.

Phenazine quantification

PZs were extracted from strain *Pseudomonas chlororaphis* subsp. *aureofaciens* DSM 6698 as described previously. Briefly, cultures were grown overnight (16 to 18 h) in LB to late exponential phase (optical density at 620 nm = 1.8) and cell-free supernatants prepared by centrifugation (2,600 g) for 15 min. Total PZs from cell-free supernatants were extracted with an equal volume of acidified benzene, and the benzene phase was separated and evaporated under air.[10] Dried PZs were dissolved in 0.1 N NaOH and quantified by UV-visible spectroscopy using 0.1 N NaOH as the blank and PCA as standard. The absorption maxima for PCA were measured at 367 nm and 484 nm respectively. The relative amounts of PCA were calculated by extrapolation of the obtained values with those of the PCA standard. We conducted three separate experiments, each over a 4-day period.[7]

Analytical high performance liquid chromatography (HPLC)

To determine the presence and concentration of phenazines, samples were analyzed by HPLC essentially according to the method of Delaney *et al*.[15] Extracts were dissolved in acetonitrile containing 0.1% trifluoracetic acid (TFA) and were separated on an Agilent 1100 series HPLC equipped with a diode array detector (DAD) G135A. The solvent system consisted of 0.1% TFA in water/acetonitrile (65:35) which was pumped through a column ACE C18, 250 × 4.6 mm in diameter (Jones Chromatography, UK) at a flow rate of 0.7 ml/min. Phenazine compounds were recognised by their retention times and UV spectra, which were compared with those of an authentic sample of PCA by superimposition. They were also quantified by comparison of areas under the peaks with those given by the authentic PCA standard.[15,16]

Analysis of phenazine

GC-MS analysis of the crude extracts were undertaken on a Hewlett Packard 6890 electrospray mass spectra with quadrupole mass spectrometer model 5973. This analysis was performed on a 30-m HP5MS capillary column of internal diameter 0.25 mm and 0.25 μm film thicknesses. Crude extracts (5 μl) in methanol were injected into a column oven temperature of 60 °C; after 1 min, a temperature ramp of 6 °C min^{-1} increased the column oven temperature to 280 °C. Electron impact (EI) mass spectra (70 eV) of the parent compounds were recorded. In the splitless mode, spectra were scanned at a speed of 10 s for m/z 50 to 450. Spectra were recorded and processed with the NIST software. [17]

Antimicrobial assays

After visualization on TLC, the 8 obtained spots were scraped, the silica gel removed and the methanol solutions were tested for antimicrobial activity, after concentration by evaporation in order to determine which are active. Antimicrobial activities of material extracted from the culture into ethyl-acetate were determined by the paper disc agar diffusion bioassay method. *E. coli* MC 4100 NCTC 9002, *Bacillus subtilis*, *Pseudomonas diminutus* NCTC

8545, *Paracoccus paratrophus*, *Micrococcus luteus* and *Salmonella enteridis* were used as test organisms. 20 µl (containing 20 µg of crude extract and equivalent to 0.31 ml culture filtrate) in methanol were evaporated on sterile paper discs (6 mm diameter, Whatman paper Grade AA) and applied to the MH agar already inoculated with 100 µl of the sensitive bacteria. Plates were stored overnight at 4 °C, and then incubated at 37 °C for 24 h.[18]

RESULTS

Identification

After chromatographic separation on TLC plates under UV light (Table 1), the bio-activity of the extracts of *Pseudomonas chlororaphis* DSM 6689 was confined to 3 spots. These spots were also identified according to their color appearance, UV absorbance, UV and visible spectra and HPLC spectra.

The compound with an R_f of 0.20 with highest activity, which was identified as phenazine-1-carboxylic acid (PCA) according to its yellow color, dark appearance under UV (absorbed UV light) and spectral characteristics. Its UV spectrum in NaOH 0.1 N gave a strong peak at 248 and a broad peak at 365 nm with a shoulder at 354 nm. The superimposition of the HPLC spectra with the authentic sample of this spot gave as much of 99% similitude (Figure 1).

A second spot with an R_f of 0.30 with less activity, tentatively identified as 2-OH-PCA on the basis of its orange appearance and orange fluorescence under UV light. It has UV absorption maxima at 269 and 485 nm, and a weaker peak at 290 nm.

The third spot with an R_f of 0.08 with minor activity, was tentatively identified as 2-OH-PHZ (2-hydroxy phenazine) according to its lightly brownish appearance, also fluoresced orange under UV light. The superimposition of these UV-visible spots with the sample of the referenced strain 30–84 gave a perfect much (Figure 2).

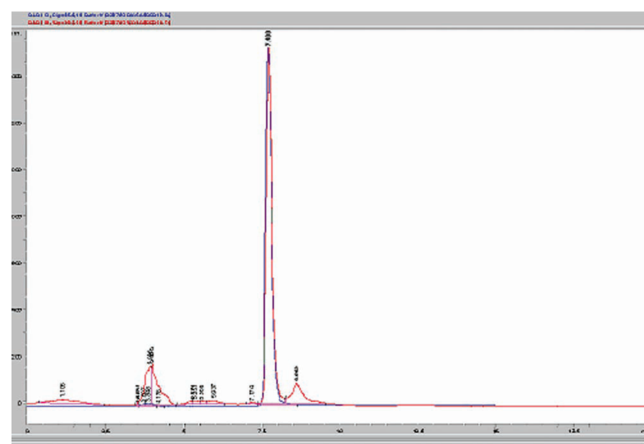

Figure 1. HPLC superimposition of PCA Standard (blue) and strain *Pseudomonas chlororaphis* subsp. *aureofaciens* DSM 6698 extracts (red) spectra.

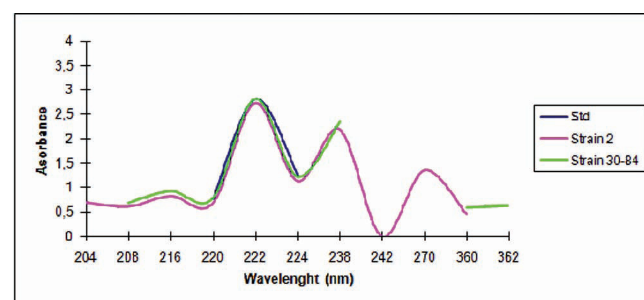

Figure 2. Superimposition of UV-Visible spectra of PCA Standard (black), Strain 2: *Pseudomonas chlororaphis* subsp. *aureofaciens* DSM 6698 extracts (Purple) and Strain 30–84: *Pseudomonas chlororaphis* 30–84 extracts (green).

Further analysis by HPLC yielded a symmetrical peak detected at 248 nm as previously reported, with a retention time of 7.69 min which corresponded to the PCA standard, another peak with a retention time of 8.59 min which corresponded to 2-OH-PCA, and a third peak with a retention time of 3.97 min which corresponded to 2-OH-PHZ (by comparing our peaks to the UV visible superimposition spectrum of the strain 30–84).

Table 1: Spots obtained after TLC under UV light

Spots	R_f	Color	Color Under UV light	Activity
1	0.04	brown	yellow	–
2	**0.08**	**Light brown**	**orange**	+
3	0.11	Light brown	blue	–
4	0.14	yellow	green	–
5	0.17	pink	blue	–
6	0.18	Light brown	yellow	–
7	**0.2**	**yellow**	**dark**	+++
8	**0.3**	**orange**	**orange**	++

R_f: frontal report; + and –: positive or negative activity; +++: highest activity; ++: less activity.

Phenazine residues in the methanol extract was also confirmed by determining the mass spectrum. This mass spectrum gave three peaks, including the most abundant compound is one having m/z 180 (M + –CO2) corresponding to PZ, which it is coupled with the existence of a fragment ion m/z 154 corresponding to the PCA and another fragment ion (m/z 196) was found corresponding to the 1-OH-PHZ. These two were poorly separated. These are probably the two isomers of phenazine molecule.

Antimicrobial activity

The benzene fraction of *Pseudomonas chlororaphis* subsp. *aureofaciens* DSM 6698 supernatants completely inhibited the growth of *R. solani*; *P. ultimum* and *F. oxysporum* at 60, 40 and 20 µg/ml of the active extract, as well as its three active spots (data not shown). The crude bacterial extracts, 20 µg and 10 µg, also inhibited the growth of *Bacillus subtilis*, *Paracoccus paratrophus*, *Pseudomonas diminutus* and *Micrococcus luteus* with zones of inhibition of 15 mm, 25 mm, 8 mm, and 3 mm respectively. The antimicrobial activity against *Salmonella enteridis* was not quantified.

DISCUSSION

The crude extracts of *Pseudomonas chlororaphis* DSM 6689 were active against the tested microorganisms (Gram positive bacteria and fungi). The active metabolites were identified tentatively as phenazines (PCA, 2 OH-PCA and 2OH PHZ), on the basis of their color under UV light, UV spectra, R_f on TLC, retention time in HPLC, and Mass spectrum .

The identification of the three spots was in accordance with many references. The most active one which was identified as phenazine-1-carboxylic acid (PCA) is as previously described by Pierson and Thomashow,[11] Delanay *et al*.,[15] El-Sayed *et al*.,[16] and Gerber.[19]

The second one corresponded to the 2-hydroxy-PCA. as previously described by Pierson and Thomashow,[11] Delaney *et al*.,[15] and Veselova *et al*.[20]

The third spot was tentatively identified as 2-OH-PHZ (2-hydroxy phenazine)[11,15,20] according to Delanay *et al*.,[15] and Mann.[21] Phenazine derivatives produced by *Pseudomonads* can easily be extracted from supernatants of spent cultures and analyzed using HPLC and with diode array detection.[22,23]

The HPLC yielded similar results to those obtained by Delaney *et al*.,[15] Veselova *et al*.,[20] Bonsall et al.,[24] Mavrodi *et al*.,[25] and Liu *et al*.[26]

The mass spectrum of Phenazine residues in the methanol was comparable to those obtained by Saosoong *et al*.,[17] Gurusiddaiah *et al*.,[27] Brisbane *et al*.,[28] and Glandorf *et al*.[29]

The production of PCA by *Pseudomonas chlororaphis* DSM 6689 was about 0.05 mg from 0.78 ml of culture filtrate. Bakker *et al*.,[30] demonstrated that the production of PCA increased antifungal activity (against soil wheat microflora as *Gaeummanomyces graminis* var. *tritici*) of *Ps.putida* and 2, 4-diacetyl phloroglucinol (DAPG) production resulted in an enhanced ability to inhibit growth of both fungi and bacteria.

The crude extract was more active against the phytopathogenic fungi than its compounds, when these compounds were tested separately, each one independently. May be this is due to a synergic effect of the different compounds).

Delaney *et al*.,[15] evaluate the importance of hydroxylated phenazines in the biological control activity against *Gaeummanomyces graminis* var. *tritici*, and demonstrated that the ability to produce hydroxyphenazine compounds was correlated with greater antifungal activity than was production of PCA alone. *Pseudomonas spp.* produces phenazine antibiotics - nitrogen-containing heterocyclic pigments, which exhibit broad-spectrum activity against numerous bacteria and fungi.[1,31,32]

Veselova *et al*.,[20] demonstrated that extracts of *Pseudomonas chlororaphis* 449 which produces phenazines (PCA, 2 OH-PCA and 2OH PHZ) inhibited the growth of the Gram +ve bacteria, *Bacillus subtilis* and *Staphylococcus aureus.* More than 6000 phenazine-containing compounds have been identified and reported during the last two centuries, including synthetic analogues; fewer than one hundred are of natural origin, with some of the *Pseudomonas spp*. synthesizing a mixture of phenazine derivatives.[26]

The most commonly identified derivatives produced by *Pseudomonas spp.* are pyocyanin, PCA, and a number of hydroxy-phenazines.[1,32] These compounds also had antibacterial activity[20] but were not inhibitory to yeasts.[33]

The ability to produce hydroxyphenazine compounds is correlated with greater antifungal activity than production of PCA alone.[15] Smirnov and Kiprianova[31] found that 2-hydroxyphenazine exhibited strong bacteriostatic and fungistatic activity against a variety of bacterial (Gram-positive and Gram negative) and fungal animal and plant pathogens, than PCA and 2-OH-PCA.

Phenazine-1-carboxylic acid, also known as tubermycin B because of its antibiotic activity against *Mycobacterium*

tuberculosis, is believed to be one of the metabolic precursors for other phenazines like 2 OH PCA, 2 OH PZ or more complex phenazine metabolites.[28,34]

Tubermycin B efficiently inhibited growth of *Bacillus cereus* (MIC 0.5 μg/ml) but showed only modest antibiotic activity against *Micrococcus luteus* and *Staphylococcus aureus* (MIC 5 μg/ml).[34]

The mechanisms for the action of phenazines interactions are poorly understood. It is assumed that they diffuse across or insert into the membrane and act as a reducing agent, resulting in the uncoupling of oxidative phosphorylation and the generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to the cell (which over-whelm cellular superoxide dismutases and ultimately cause cell death).[1,15,35–38] Krishnan *et al*.,[32] demonstrate that transmission electron micrographs of nodules of *Rhizobium etli* USDA 9032 initiated by phenazine-producing rhizobia clearly reveal the loss of membrane integrity, due to the free radicals generated by rapid oxidation and reduction undergone by phenazine compounds. These subsequently result in cell death. Their observations were consistent with the role of reactive oxygen species in nodule senescence observed earlier by Matamoros *et al*.,[39] leading to oxidative damage of macromolecules.

The design of synthetic phenazines ideally strives to improve known biological activities like antibiotic, antitumor, antimalaria, and antiparasitic activities, which are results of cell growth inhibition or even cell death.[4] The physiological function leading to this activity can be inhibition/control of DNA, RNA, and protein synthesis as well as disruption of energy- requiring membrane-associated metabolic processes.[4] Possible modes of action thus include DNA inter-action (intercalation or groove binding), topoisomerase interaction, and the role of phenazines as antioxidants or charge-transferring molecules.[11]

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