Pesticide Biochemistry and Physiology 117 (2015) 39–46

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effects of phenazine-1-carboxylic acid on the biology of the plant-pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae*

Shu Xu [a,b,1], Xiayan Pan [a,b,1], Jianying Luo [a,b], Jian Wu [a,b], Zehua Zhou [a,b], Xiaoyu Liang [a,b], Yawen He [c], Mingguo Zhou [a,b,*]

[a] College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China
[b] Key Laboratory of Pesticide, Nanjing Agricultural University, Jiangsu Province, China
[c] State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai, China

ARTICLE INFO

Article history:
Received 7 February 2014
Accepted 6 October 2014
Available online 16 October 2014

Keywords:
Phenazine-1-carboxylic acid
Xanthomonas oryzae pv. *oryzae*
Biological effects

ABSTRACT

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the casual agent of bacterial blight, which is one of the most serious diseases of rice. The antibiotic phenazine-1-carboxylic acid (PCA), which is primarily produced by *Pseudomonas* spp., was found and previously reported very effective against *Xoo*. However, the biological effects of PCA on *Xoo* remain unclear. In this study, we found that PCA increased the accumulation of reactive oxygen species (ROS) and reduced the activities of catalase (CAT) and superoxide dismutase (SOD) in *Xoo*. *Xoo* was more sensitive to H_2O_2 than *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*), and had a much lower expression of CAT genes. In addition, proteomic analysis indicated that PCA inhibited carbohydrate metabolism and nutrient uptake in *Xoo*, and analysis of carbon source utilization further confirmed that carbohydrate metabolism in *Xoo* was repressed by PCA. In conclusion, PCA acted as a redox-cycling agent that disturbed the redox balance in *Xoo* and reduced CAT and SOD activities, resulting in higher accumulation of ROS, altered carbohydrate metabolism, and lower energy production and nutrient uptake. Moreover, a deficient antioxidant system in *Xoo* made it very sensitive to PCA.

1. Introduction

Phenazine-1-carboxylic acid (PCA), an antibiotic that is primarily produced by *Pseudomonas* spp., inhibits many microorganisms' growth including various plant pathogens [1,2]. With the common name of shenqinmycin, PCA has received a Pesticide Registration Certification from the Ministry of Agriculture in China against *Fusarium* wilt infecting watermelon, *Phytophthora* blight on pepper and sheath blight on rice because of its high efficiency against various plant pathogens, low toxicity to humans, and environmental compatibility [3]. PCA belongs to the phenazine family of compounds, which because of their redox activity play important roles in iron acquisition, respiration, redox balancing, signaling, and community development in their producers; in addition, phenazine producing microorganisms possess multiple mechanisms that are likely to increase their tolerance to potentially toxic compounds and to increase their superoxide dismutase activity [4,5]. However, phenazines generate reactive oxygen species (ROS) [6], leading to cell death in organisms lacking such tolerance mechanisms.

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the casual agent of bacterial blight of rice, which is a serious disease that can reduce rice yields of susceptible varieties by as much as 50% under favorable conditions [7]. Currently, use of resistant cultivars of rice is the major method that is employed to control this disease, but bactericide application is much to be explored as a viable option [8,9]. Although bismerthiazol and streptomycin are commonly used to control bacterial blight of rice in China, *Xoo* has developed resistance to both of these bactericides [10,11]. Thus, new bactericides are required to be explored for control of *Xoo*. In an earlier study, we found that PCA was very effective against *Xoo* both *in vitro* (baseline sensitivity was assayed) and *in vivo* [12]. Although phenazine compounds have been reported to affect a wide variety of fungi and gram-positive bacteria [1,2,13–17], some phenazine compounds like clofazimine have been reported ineffective against gram-negative bacteria [18]. However, we found that PCA is very effective against *Xoo*, which is a gram-negative bacterium.

Multiple mechanisms of inhibition have been reported for phenazine compounds, including inhibition of K^+ transport [19–21], binding to DNA [22,23], inhibition of nitric oxide prodrug action [24], inhibition of RNA synthesis [14], and disturbance of the redox balance and electron transport of the respiratory chain [25,26]. However, the biological effects of PCA on *Xoo* remain unclear. In the present study, we exploited biochemical and proteomic methods to investigate how PCA affects *Xoo*.

* Corresponding author. College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China. Fax: +86 025 84395641.
E-mail address: mgzhou@njau.edu.cn (M. Zhou).
[1] Joint first authorship.

http://dx.doi.org/10.1016/j.pestbp.2014.10.006
0048-3575/

2. Materials and methods

2.1. Bactericides, bacterial strains, and culture conditions

PCA (98%) was provided by Shanghai Nongle Biological Products Co., Ltd (China). For addition to cultures, PCA was dissolved in acetone as indicated. PCA concentrations used in different assays were optimized according to the original bacterial concentrations. Wide-type ZJ173 strain of *Xoo* and RS105 strain of *Xoc*, that are commonly used in China, were used in this study. ZJ173 or RS105 were grown at 28 °C in nutrient broth (NB) medium in flasks or on nutrient broth agar (NA) medium in plates as described previously [10]. NA medium was prepared with 3 g of beef extract, 5 g of polypeptone, 1 g of yeast extract, 10 g of sucrose, and 15 g of agar powder per liter of distilled water. NB medium contained the same components but lacked agar powder. XCM medium (minimal medium) was prepared with 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 20 mM succinic acid, and 0.15% casein acid hydrolysates per liter of distilled water.

2.2. ROS accumulation in Xoo as affected by PCA

ROS accumulation was measured with a ROS assay kit (Beyotime, China) according to the instruction manual. In brief, bacteria cultures at late-log phase were collected by centrifugation (6000 × *g*, 10 min, room temperature) and resuspended in 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (1:1000 diluted with XCM medium). The bacterial suspensions were then incubated at 37 °C for 20 min in the centrifuge tubes, which were inverted every 5 min. After extracellular DCFH-DA was removed by centrifugation (6000 × *g* for 10 min at room temperature, three times with XCM medium washes), the cells were resuspended in XCM medium and added to microplates. The wells were treated with an acetone solution with or without PCA to achieve final PCA concentrations of 0, 1, or 2 mg/L and a final acetone concentration of 0.4% (v/v). As a positive control, the XCM medium contained Rosup (a compound mixture, provided by the assay kit). As a negative control, the XCM medium contained no agent (no acetone, PCA, or Rosup). The microplates were kept at 28 °C, and a Spectra Max M5 fluorescence microplate reader (Molecular Devices Corp.) was used to record the fluorescence for 4 h with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Each treatment was represented by three replicates, and the experiment was conducted three times.

2.3. Activities of superoxide dismutase (SOD) and catalase (CAT) in Xoo as affected by PCA

ZJ173 was cultured to early-log phase (OD_{600} ≈ 0.2) in NB medium, which was then supplemented with acetone solution without or with PCA to achieve a final PCA concentration of 0 or 2 mg/L and a final acetone concentration of 0.4% (v/v). Bacterial cells were collected after incubation at 28 °C for 1, 2, 4, and 8 h, and were washed with precooled 0.1 M PBS (pH 7.8). The washed bacteria were lysed by grinding in liquid nitrogen and resuspended in precooled 0.1 M PBS (pH 7.8). The cell debris were removed by centrifugation (8000 × *g* for 10 min at 4 °C), and the protein concentrations were determined with the Bradford protein assay kit (Sangon Biotech, China). Enzyme activities of SOD and CAT were assayed and calculated with SOD assay kit (Total SOD) and CAT assay kit (visible spectrophotometry) according to the instruction manual (Nanjing Jiancheng Bioengineering Institute, China). The absorption values were recorded with a Spectra Max M5 fluorescence microplate reader (Molecular Devices Corp., USA). Each treatment was represented by three replicates, and the experiment was conducted three times. The results were analyzed with SPSS 20.0 (independent-samples *t*-test).

2.4. Sensitivity of Xoo and Xoc to H_2O_2

ZJ173 or RS105 was cultured to OD_{600} ≈ 1.0 in NB medium. The bacterial suspension was diluted 3 fold or 9 fold with NB medium. After that, 5 μL of undiluted, one third, or one ninth bacterial suspension was spotted onto NA plates containing 0 mM, 0.1 mM, 0.25 mM, 0.5 mM or 0.75 mM H_2O_2. The plates were incubated at 28 °C until the diameter of undiluted bacterial colony on 0 mM H_2O_2 plate reached 1 cm. Each treatment was represented by three replicates, and the experiment was repeated three times.

2.5. CAT genes expression level of Xoo and Xoc

ZJ173 or RS105 was cultured to OD_{600} ≈ 0.5 in NB medium. The bacterial cells were collected by centrifugation (12,000 × *g* for 2 min at 4 °C). Total RNA was extracted with RNAprep pure cell/bacteria kit (TIANGEN Biotech, Beijing, China) and reverse transcribed into cDNA with PrimeScript RT reagent kit (TaKaRa, Japan) according to the instruction manual. Three CAT genes were tested, including XOO0417/XOC_4325, XOO0447/XOC_4290, and XOO3423/XOC_1265, and *gyrB* was used as reference gene. qRT-PCR was conducted with Applied Biosystems 7500 Real-Time PCR System (Life technologies, USA) using SYBR green supermix (Bio-Rad, USA). Primers used in this study are listed in Supplementary Table S2.

2.6. The proteome of Xoo as affected by PCA

Protein samples were prepared as described previously with modification [12]. In brief, after cells were cultivated to the early-log growth phase (OD_{600} ≈ 0.2) at 28 °C with shaking at 175 rpm, the cultures were treated with acetone without or with PCA to achieve a final PCA concentration of 0 or 2 mg/L and a final acetone concentration of 0.4% (v/v). After incubation for 4 h at 28 °C, bacterial cells were harvested and washed thoroughly with 10 mM PBS (pH 7.4). The washed bacteria were lysed by sonication (with cooling on ice) with lysis buffer containing 8 M urea, 2 M thiourea, 65 mM DTT, 4% (w/v) CHAPS, 0.2% (w/v) Bio-Lyte (pH 3–10), 0.001% (w/v) bromophenol blue, 1 mM PMSF, and 2 mM EDTA. The unbroken cells and cell debris were removed by centrifugation (25,000 × *g* for 1 h at 4 °C), and the supernatant was further purified with a 2-D cleanup kit according to the instruction manual (Bio-Rad, USA). The proteins were collected and dissolved in lysis buffer as described above. Protein concentrations were determined with the Bradford protein assay kit (Sangon Biotech, China).

Immobilized pH gradient (IPG) strips (Bio-Rad, USA; 17 cm, pH 4–7) were passively rehydrated with approximately 150 μg of proteins in 300 μL of lysis buffer; the strips were incubated for 16 h at 20 °C. Isoelectric focusing (IEF), equilibration, and SDS-PAGE were performed as described previously [12]. The gels were stained with a modified silver stain method [27]. The stained gels were then scanned with a GS-800 scanner (Bio-Rad, USA). Three biological and three analytical replicates were conducted, and the spots were analyzed with PDQuest 8.0 software (Bio-Rad, USA). The spot volume was normalized with the local regression model, and spots with fold-changes ≥1.5 (Student's *t*-test, $p < 0.05$) were considered differentially expressed. Most of the differentially expressed proteins could be found in the *Xoo* proteome reference database established previously in our laboratory [12]. The unmatched spots were digested with trypsin and identified with MALDI-TOF/TOF MS as described previously [27].

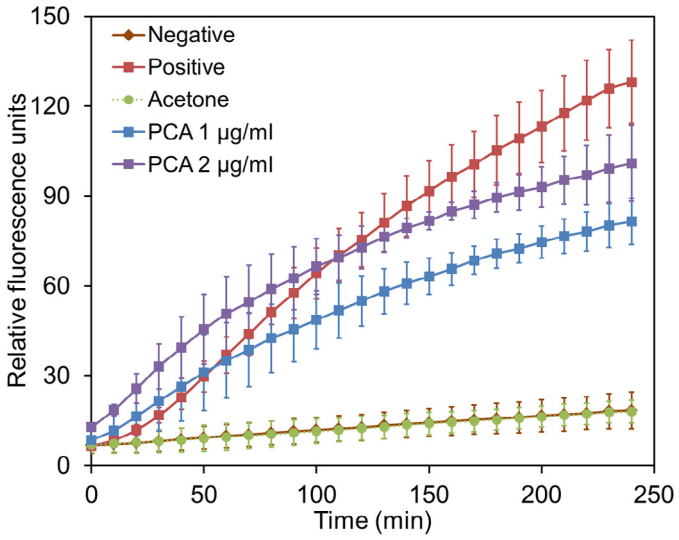

Fig. 1. ROS accumulation in *Xoo* as affected by PCA. The treatments included PCA in acetone, acetone alone (a negative control), Rosup (a positive control), or none of these agents (a negative control). Values are means and standard deviations from three independent experiments.

2.7. Utilization of carbon sources by Xoo as affected by PCA

All the materials and instruments used in this experiment were purchased from Biolog Co., Ltd. (USA). Cells from a single colony of ZJ173 growing on NA were inoculated into inoculating fluid A with a sterile cotton tipped swab, and the turbidity was adjusted to 90–98% with a turbidimeter. As before, acetone alone or with PCA was added to the inoculating fluid A to obtain a final PCA concentration of 0 or 0.2 mg/L and a final acetone concentration of 0.4% (v/v). Lower PCA concentration (0.2 mg/L) was used in this assay because the original bacterial concentration was much lower than other experiments. The suspension was then added to the Gene III microplates with a multichannel pipette (100 μL each well). The microplates were kept moist with moistened paper and incubated at 28 °C for 7 days. The absorption values at 590 nm and 750 nm were recorded with the Gen III Microstation every 24 h. The average well color development (AWCD) was calculated with the formula $\Sigma(C_i - R)/n$, in which C_i stands for OD_{590}–OD_{750} of each calculated well, R stands for the C value of the negative control A1, and n is the number of calculated wells; for wells in which $C_i - R < 0$, AWCD was recorded as 0. This experiment was performed three times. The data set was analyzed with SPSS 20.0 (independent-sample *t*-test).

3. Results

3.1. ROS accumulation in Xoo as affected by PCA

As evident from Fig. 1, ROS accumulated in *Xoo* when treated with PCA or Rosup (the positive control), and the accumulation was greater with 2 mg/L of PCA than with 1 mg/L of PCA. However, ROS accumulation was not detected in the negative control or acetone treatment.

3.2. Activities of superoxide dismutase (SOD) and catalase (CAT) in Xoo as affected by PCA

As shown in Fig. 2, SOD and CAT activities in *Xoo* were found repressed by PCA at all sampling times except for SOD activity at 2 h and CAT activity at 8 h. No significant difference was found between the two treatments for SOD activity at 2 h and CAT activity at 8 h. Interestingly, CAT activities were found much lower in *Xoo* than in plants or other *Xanthomonas* pathogens (data not shown).

3.3. Sensitivity of Xoo and Xoc to H_2O_2

As shown in Fig. 3, *Xoo* was found extremely sensitive to H_2O_2, and didn't grow even at 0.1 mM. In contrast, *Xoc* was much more tolerant to H_2O_2. It grew well at 0.5 mM and also grew quite well at 0.1 mM even if diluted nine fold.

3.4. CAT genes expression level of Xoo and Xoc

Three CAT gene (XOO0417/XOC_4325, XOO0447/XOC_4290, and XOO3423/XOC_1265) expression levels in *Xoo* and *Xoc* were determined by qRT-PCR and it was found that all three genes showed higher expression levels in *Xoc* than *Xoo* (Fig. 4). Interestingly, XOC_4325 and XOC_1265 expressed more than 11 fold and 3 fold higher in *Xoc*, respectively.

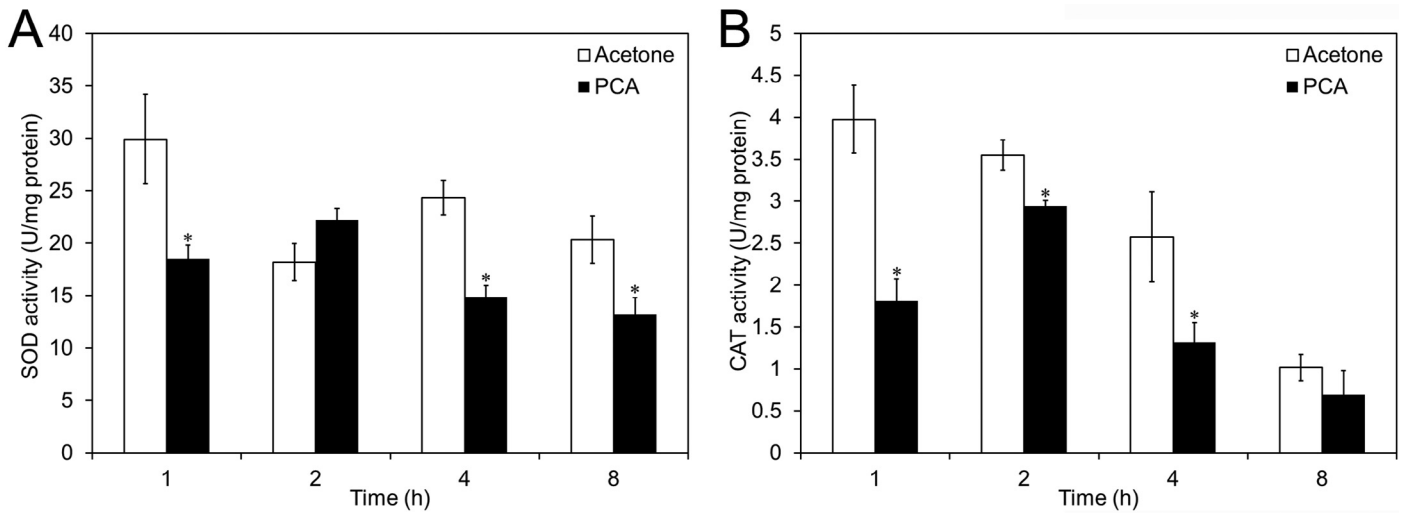

Fig. 2. SOD (A) and CAT (B) activities of *Xoo* as affected by PCA. The treatments included PCA in acetone and acetone alone. Values are means and standard deviations from three independent experiments. Statistical differences were detected with independent-samples *t*-tests.

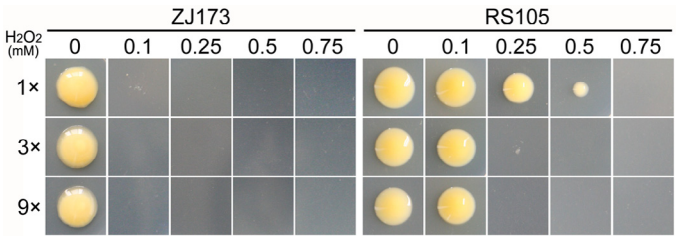

Fig. 3. Sensitivity of *Xoo* and *Xoc* to H_2O_2. Bacterial suspension was spotted on NA plates containing different concentrations of H_2O_2. Photographs were taken when the diameter of undiluted bacteria colony on 0 mM H_2O_2 plates reached 1 cm.

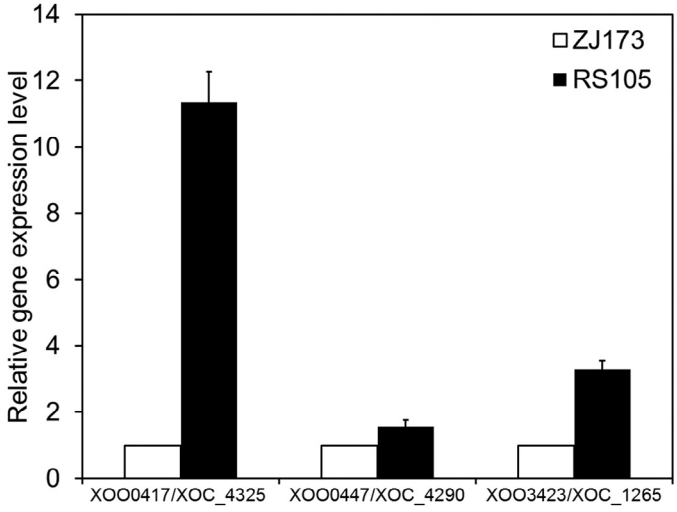

Fig. 4. CAT gene expression level in *Xoo* and *Xoc*. Three CAT genes (XOO0417/XOC_4325, XOO0447/XOC_4290, and XOO3423/XOC_1265) were tested with qRT-PCR.

3.5. The proteome of Xoo as affected by PCA

A total of 43 protein spots were differentially expressed by *Xoo* that was treated or not treated with PCA (Fig. 5). The 43 spots included 40 that were down-regulated and three that were up-regulated (Table 1). Of the 43 spots, 37 were found in the *Xoo* proteome reference database established previously in our laboratory, and the six unmatched spots were identified with MALDI-TOF/TOF MS (Supplementary Table S1). Of the differentially expressed protein spots, >30% are involved in carbohydrate metabolism, and 27.9% are transportation-related proteins (Fig. 6).

3.6. Utilization of carbon sources by Xoo as affected by PCA

The Gene III MicroPlate consists of 71 carbon sources, 23 chemical-sensitive assays, one negative control, and one positive control. The 71 carbon sources are classified as carbohydrates, amino acids, alcohols, hexose acids, and carboxylic acids, esters, and fatty acids. Treatment of *Xoo* with PCA reduced the overall AWCD from day 3 through day 7 (Fig. 7A). On day 7, PCA had significantly reduced the AWCDs for all carbon sources, carbohydrates, and carboxylic acids, esters, and fatty acids (Fig. 7B); however, PCA had not significantly changed the AWCDs for amino acids and had significantly increased the AWCDs for hexose acids and alcohols (Fig. 7B). Among the 71 carbon sources, the AWCDs of 15 species were >0.1 and were changed by ≥1.5-fold when treated with PCA (Fig. 7C). The AWCDs of the 23 chemical-sensitive assays were not significantly changed by PCA treatment, and no AWCD among them was >0.1 and changed by ≥1.5-fold.

4. Discussion

As noted earlier, the antibiotic PCA belongs to the phenazine family of compounds, which have been reported to be effective against many fungi and gram-positive bacteria but not against gram-negative bacteria [1,2,13–15,18]. Interestingly, we previously found that PCA is very effective against *Xoo*, which is a gram-negative bacterium [12]. However, we also found that PCA is not very effective against *Xoc*, and ineffective against *Xanthomonas campestris* pv. *campestris* and

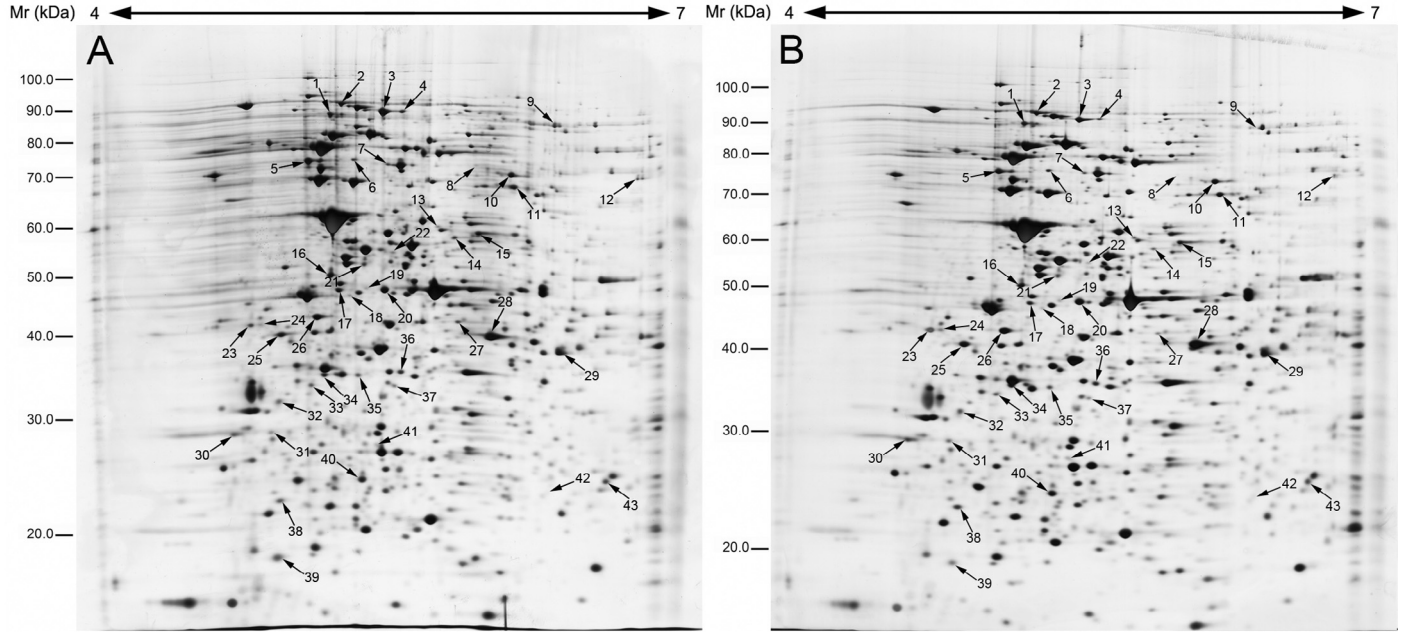

Fig. 5. 2DE maps of *Xoo* treated with PCA (B) or without PCA (A). PCA treatment caused a ≥1.5-fold difference in expression for 43 protein spots (Student's *t*-test, $p < 0.05$).

Table 1
Differentially expressed proteins in *Xoo* treated with acetone solution of PCA or acetone alone.

Spot	Fold change[a]	Protein description[b]	GI in NCBI	Functional category
1	–1.61	TonB-dependent receptor	84623344	Transportation
2	–1.68	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	58582485	Carbohydrate metabolism
3	–2.76	TonB-dependent receptor	58580573	Transportation
4	–1.99	30S ribosomal protein S1	58581803	Translation
5	–1.54	TonB-dependent outer membrane receptor	188578441	Transportation
6	–1.88	Two-component system regulatory protein	122879024	Signal transduction
7	–1.65	Isocitrate dehydrogenase	58583790	Carbohydrate metabolism
8	–2.41	Iron transporter	58424234	Transportation
9	–1.9	Pyruvate dehydrogenase subunit E1	84625667	Carbohydrate metabolism
10	–1.91	Transketolase	58580799	Carbohydrate metabolism
11	–1.61	Dihydrolipoamide dehydrogenase	58424939	Carbohydrate metabolism
12	–1.7	Ferrichrome-iron receptor 3	58580249	Transportation
13	1.53	Phosphomannose isomerase; GDP-mannose pyrophosphorylase	58580419	Carbohydrate metabolism
14	–1.65	Fumarate hydratase	58581959	Carbohydrate metabolism
15	–1.52	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	58580162	Nucleotide metabolism
16	–2.58	Outer membrane protein	58584205	Transportation
17	–2.82	Phosphoglucomutase; phosphomannomutase	58580420	Carbohydrate metabolism
18	–2.79	Polyphosphate-selective porin O	58582083	Transportation
19	–3.11	Phosphoglucomutase	21230101	Carbohydrate metabolism
20	–1.91	Xylose isomerase	58428634	Carbohydrate metabolism
21	–3.06	Ferric enterobactin receptor	58581227	Transportation
22	–2.35	Replicative DNA helicase	58581148	Replication and repair
23	–2.06	Outer membrane protein	58583319	Transportation
24	–1.61	Outer membrane protein	58583319	Transportation
25	–1.65	3-Isopropylmalate dehydrogenase	58580563	Amino acid metabolism
26	–2.05	Phosphoglycerate kinase	58583038	Carbohydrate metabolism
27	–2.44	Regulator of pathogenicity factors	58582436	Transportation
28	–2	Initiation factor eIF-2B	58426624	Translation
29	–1.77	Fructose-1,6-bisphosphatase	58424232	Carbohydrate metabolism
30	–1.55	Hypothetical protein XOO1659	58581282	Unknown
31	–1.56	Hypothetical protein XOO4006	58583629	Unknown
32	–1.55	Chaperonin GroEL	58583911	Protein folding and degradation
33	–1.93	Beta-ketoacyl- synthase III	58425095	Lipid metabolism
34	–1.56	Phosphoribosylaminoimidazole-succinocarboxamide synthase	188575052	Nucleotide metabolism
35	–1.6	Pantoate-beta-alanine ligase	84623901	Amino acid metabolism
36	–1.61	Sugar kinase	84625406	Carbohydrate metabolism
37	–1.81	Hypothetical protein XOO4409	58584032	Unknown
38	–1.59	Outer membrane protein	58580340	Transportation
39	–1.8	Hypothetical protein XOO4199	58583822	Unknown
40	–1.51	Two-component system regulatory protein	21233365	Signal transduction
41	3.82	50S ribosomal protein L25/general stress protein Ctc	58583225	Translation
42	2.1	Endonuclease	58580820	Replication and repair
43	–1.5	Septum formation inhibitor	84624993	Replication and repair

[a] Fold-changes in *Xoo* treated with acetone solution of PCA compared with *Xoo* treated with acetone alone.
[b] Detailed information (scores, molecular weight and isoelectric point, sequence coverage, peptide sequences, and taxonomy) are shown in Table S1.

Xanthomonas axonopodis pv. *citri* (data not shown), indicating that *Xoo* may be unusual among gram-negative bacteria with respect to PCA sensitivity. Although multiple mechanisms of inhibition have been reported for several phenazine compounds, the biological effects of PCA on *Xoo* remain unclear. In this study, we investigated the effects of PCA on various aspects of *Xoo* biology.

Phenazines have been categorized as antibiotics that generate ROS [6]. In this study, a DCFH-DA fluorescence probe method was

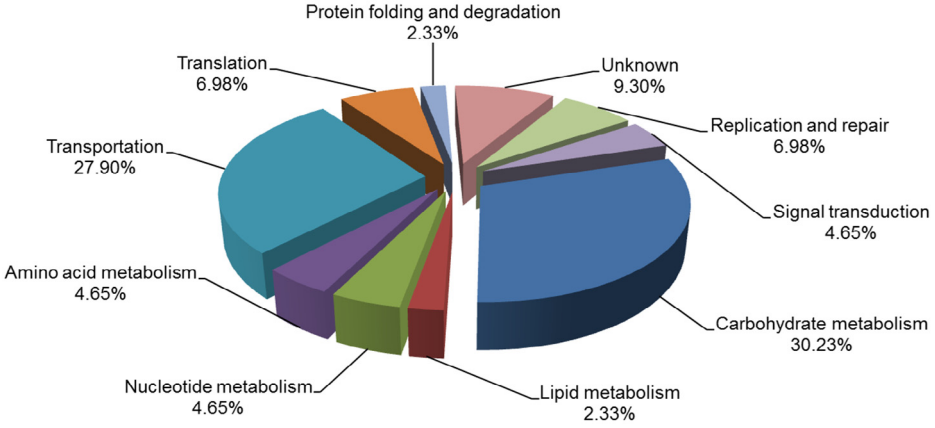

Fig. 6. Functional distribution of protein spots differentially expressed in *Xoo*. Functional categories were assigned according to the KEGG and NCBI databases.

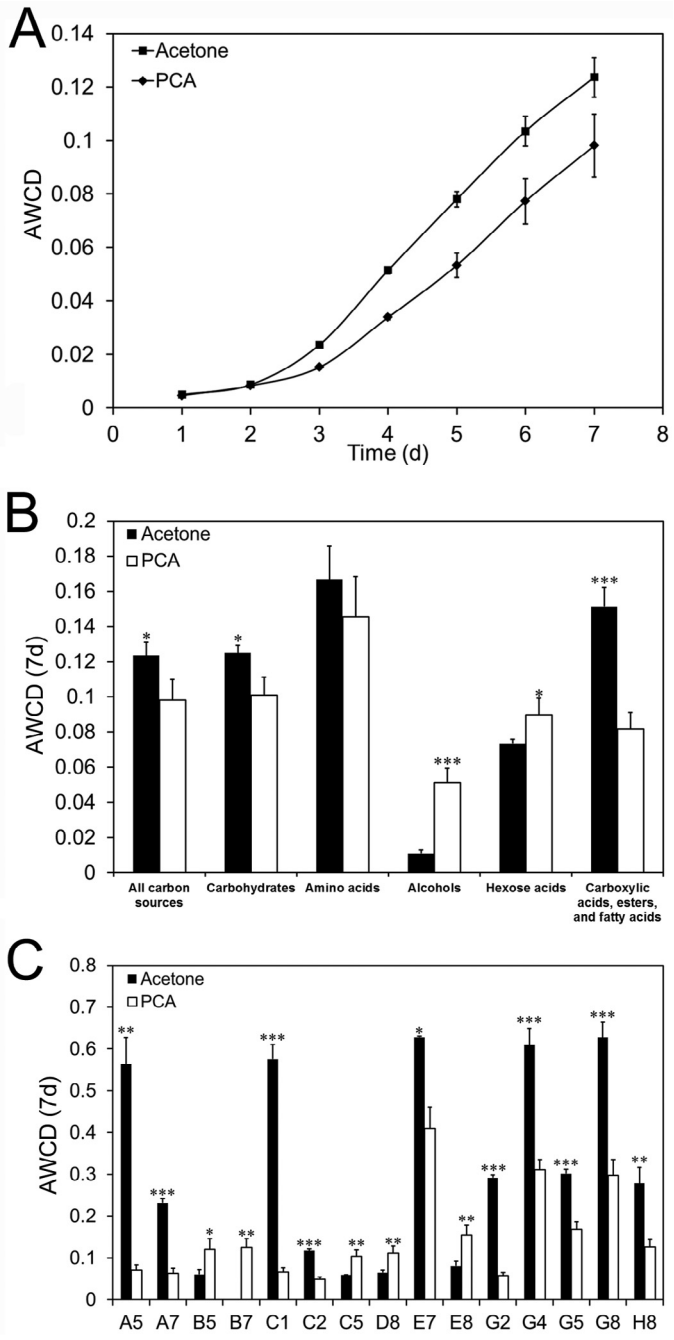

Fig. 7. Carbon source utilization by *Xoo* as affected by PCA. (A) Average well color development (AWCD) of all 71 carbon sources over time; (B) AWCDs of different groups of carbon sources on day 7; (C) AWCDs that were greater than 0.1 and that were changed by ≥1.5-fold on day 7 for particular carbon sources. A5, D-cellobiose; A7, sucrose; B5, D-salicin; B7, N-acetyl-β-D-mannosamine; C1, α-D-glucose; C2, D-mannose; C5, 3-methyl glucose; D8, D-aspartic acid; E7, L-histidine; E8, L-pyroglutamic acid; G2, methyl pyruvate; G4, L-lactic acid; G5, citric acid; G8, L-malic acid; H8, acetic acid. Values are means and standard deviations from three independent experiments. Statistical differences were detected with independent-sample *t*-tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

used to quantify ROS accumulation in *Xoo* treated by PCA. When treated with PCA or positive control Rosup, substantial ROS accumulated in *Xoo*. This result indicated that PCA, like other phenazine compounds, generates ROS in *Xoo* and that ROS generation is likely to be an important mechanism of PCA action, as is the case with the phenazines clofazimine and 5MPCA [25,26]. In addition, PCA inhibited SOD and CAT activities in *Xoo* at most sampling times, which would reduce ROS scavenging ability. Therefore, the accumulation of ROS in *Xoo* could be a combined effort of PCA redox reaction and lower ROS scavenging ability.

In CAT activities experiment, we found that CAT activities of *Xoo* were unexpectedly low. Therefore, we compared H_2O_2 sensitivity of *Xoo* with another pathovar of *Xanthomonas oryzae* known as *Xoc*, which was about 33 fold (EC_{50}) more resistant to PCA than *Xoo* (data not shown). Interestingly, *Xoo* was extremely sensitive to H_2O_2, while *Xoc* is much more tolerant. We also tested three CAT genes' expression levels both in *Xoo* and *Xoc*, and found that two of them had a much higher expression level in *Xoc*. These results indicated that *Xoo* has a deficient antioxidant system which made it very sensitive to PCA.

Proteomic analysis of *Xoo* showed that 43 protein spots were differentially expressed in response to PCA treatment. As shown in Fig. 6, more than 30% of the differentially expressed protein spots were involved in carbohydrate metabolism, and all the protein spots were down-regulated in response to PCA except for phosphomannose isomerase (spot 13). Among the down-regulated proteins, isocitrate dehydrogenase (spot 7), pyruvate dehydrogenase subunit E1 (spot 9), dihydrolipoamide dehydrogenase (spot 11), and fumarate hydratase (spot 14) are important enzymes in the tricarboxylic acid cycle; transketolase (spot 10) is an important enzyme in the pentose phosphate pathway; phosphoglucomutase (spots 17, 19) is the first enzyme in glucuronate pathway; and phosphoglycerate kinase (spot 26) is an important enzyme in glycolysis [28]. These results indicated that PCA inhibited carbohydrate metabolism in *Xoo*. In addition, transportation-related proteins accounted for nearly 28% of the differentially expressed protein spots (Fig. 6), and these protein spots were down-regulated. TonB-dependent receptor (spots 1 and 3), TonB-dependent outer membrane receptor (spot 5), iron transporter (spot 8), ferrichrome-iron receptor 3 (spot 12), and ferric enterobactin receptor (spot 21) belong to the TonB-dependent receptor family proteins, which are extremely important for the uptake of iron complexes, vitamin B12, carbohydrates, nickel, and even some antibiotics in *Xoo* [29]. Many reports have shown that the TonB-dependent transportation system is energy-dependent [30–35], and lower energy production caused by reduced carbohydrate metabolism would certainly affect this system. These results indicated that PCA also inhibited nutrient uptake by *Xoo*. Clofazimine was previously reported to inhibit K^+ uptake by mycobacteria [19,20], which is similar to our result. Moreover, membrane damage such as membrane lipid peroxidation caused by accumulation of ROS [36] would also affect the transportation system of *Xoo*. 50S ribosomal protein L25/general stress protein Ctc (spot 41) and endonuclease (spot 42) were the most up-regulated protein spots, but there is no evidence that they are associated with the mode of action of PCA.

To verify whether the carbohydrate metabolism of *Xoo* was inhibited by PCA, we assessed the ability of *Xoo* to use different carbon sources with the Gen III Microstation. The Gene III microplates include 71 carbon sources, 23 chemical-sensitive assays, one negative control, and one positive control, and carbon source utilization was evaluated based on average well color development (AWCD). PCA treatment of *Xoo* significantly reduced the overall AWCD of the 71 carbon sources but did not affect the 23 chemical-sensitive assays after 7 days of incubation, indicating that the ability of *Xoo* to utilize carbon sources was reduced by PCA, and the chemical enduring ability was not significantly affected. With respect to the groups of carbon sources, the AWCDs of carbohydrates and carboxylic acids, esters, and fatty acids were significantly reduced by PCA, while the AWCDs of amino acids were not significantly changed. Interestingly, the AWCDs of hexose acids and alcohols increased significantly when *Xoo* was treated with PCA. This is probably because *Xoo* could use these as alternative carbon sources when its ability to utilize the original carbon source was inhibited. In addition, the AWCDs of the following 15 carbon sources were greater than 0.1 and were

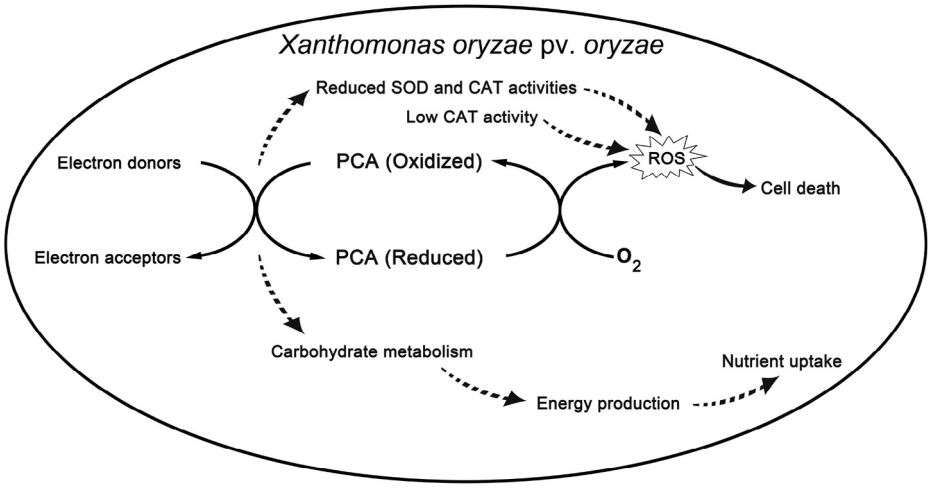

Fig. 8. A possible model for the biological effects of PCA on *Xoo*. PCA is a redox-cycling agent that can be reduced in *Xoo*. The reaction would generate a reduced form of PCA. The reduced form of PCA reacts with O_2 and generates ROS, which would damage the cell. Reduced SOD and CAT activities also contribute to the ROS accumulation. PCA would upset the redox balance of *Xoo*, which in turn disturbs carbohydrate metabolism and energy production. Lower energy production caused by reduced carbohydrate metabolism would also reduce nutrient uptake. In addition, low CAT activities in *Xoo* also made it very sensitive to PCA.

changed by ≥1.5-fold when treated with PCA: D-cellobiose, sucrose, D-salicin, N-acetyl-β-D-mannosamine, α-D-glucose, D-mannose, 3-methyl glucose, D-aspartic acid, L-histidine, L-pyroglutamic acid, methyl pyruvate, L-lactic acid, citric acid, L-malic acid, and acetic acid. Interestingly, ATP or NADH/NAD^+ is involved in the utilization of sucrose, D-cellobiose, α-D-glucose, D-mannose, D-aspartic acid, L-lactic acid, citric acid, and L-malic acid [28], suggesting that energy production may be disturbed by PCA.

PCA is a redox-cycling agent that can be reduced both *in vitro* (Supplementary Fig. S1) and *in vivo*. As shown in Fig. 8, the reaction would generate a reduced form of PCA. The reduced form PCA reacts with O_2 and generates ROS, which would damage the cell. Reduced SOD and CAT activities also contribute to the ROS accumulation. When the redox balance in *Xoo* was disturbed, carbohydrate metabolism and energy production would be reduced. Nutrient uptake would also be affected because of lower energy production caused by reduced carbohydrate metabolism and upset redox balance. In addition, low CAT activities in *Xoo* led to trouble in scavenging H_2O_2, which made it very sensitive to PCA.

Acknowledgments

This work was supported by the National Key Basic Research Program (973 Program, 2009CB118906); the National Natural Science Foundation of China (No. 31272065); the Jiangsu Province Postgraduate Innovative Project (CXZZ12_0292); and the Opening Project of State Key Laboratory of Microbial Metabolism (MMCKF13-02). We thank Qiuxia Wang, Hongyu Ma, and Ling Tang for technical assistance in this study.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.pestbp.2014.10.006.

References

[1] G. Puopolo, M. Masi, A. Raio, A. Andolfi, A. Zoina, A. Cimmino, et al., Insights on the susceptibility of plant pathogenic fungi to phenazine-1-carboxylic acid and its chemical derivatives, Nat. Prod. Res. 27 (2012) 956–966.
[2] S. Compant, B. Duffy, J. Nowak, C. Clément, E.A. Barka, Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects, Appl. Eviron. Microbiol. 71 (2005) 4951–4959.
[3] L.L. Yuan, Y.Q. Li, Y. Wang, X.H. Zhang, Y.Q. Xu, Optimization of critical medium components using response surface methodology for phenazine-1-carboxylic acid production by *Pseudomonas* sp. M-18Q, J. Biosci. Bioeng. 105 (2008) 232–237.
[4] C. Okegbe, H. Sakhtah, M.D. Sekedat, A. Price-Whelan, L.E. Dietrich, Redox eustress: roles for redox-active metabolites in bacterial signaling and behavior, Antioxid. Redox Signal. 16 (2012) 658–667.
[5] D.J. Hassett, L. Charniga, K. Bean, D.E. Ohman, M.S. Cohen, Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase, Infect. Immun. 60 (1992) 328–336.
[6] C. Jacob, V. Jamier, L.A. Ba, Redox active secondary metabolites, Curr. Opin. Chem. Biol. 15 (2011) 149–155.
[7] T.W. Mew, Current status and future prospects of research on bacterial blight of rice, Annu. Rev. Phytopathol. 25 (1987) 359–382.
[8] J.U. Jeung, S.G. Heu, M.S. Shin, C.M. Vera Cruz, K.K. Jena, Dynamics of *Xanthomonas oryzae* pv. *oryzae* populations in Korea and their relationship to known bacterial blight resistance genes, Phytopathology 96 (2006) 867–875.
[9] Y. Xu, Q.Q. Luo, M.G. Zhou, Identification and characterization of integron-mediated antibiotic resistance in the phytopathogen *Xanthomonas oryzae* pv. *oryzae*, PLoS ONE 8 (2013) e55962.
[10] X.F. Zhu, Y. Xu, D. Peng, Y. Zhang, T.T. Huang, J.X. Wang, et al., Detection and characterization of bismerthiazol-resistance of *Xanthomonas oryzae* pv. *oryzae*, Crop Prot. 47 (2013) 24–29.
[11] Y. Xu, X.F. Zhu, M.G. Zhou, J. Kuang, Y. Zhang, Y. Shang, et al., Status of streptomycin resistance development in *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola* in China and their resistance characters, J. Phytopathol. 158 (2010) 601–608.
[12] S. Xu, J.Y. Luo, X.Y. Pan, X.Y. Liang, J. Wu, W.J. Zheng, et al., Proteome analysis of the plant-pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae*, Biochim. Biophys. Acta 2013 (1834) 1660–1670.
[13] S.R. Giddens, D.C. Bean, Investigations into the *in vitro* antimicrobial activity and mode of action of the phenazine antibiotic D-alanylgriseoluteic acid, Int. J. Antimicrob. Agents 29 (2007) 93–97.
[14] L.H. Pal'chykovs'ka, I.V. Aleksieieva, V.H. Kostina, M.O. Platonov, V.V. Nehruts'ka, O.M. Deriabin, et al., New amides of phenazine-1-carboxylic acid: antimicrobial activity and structure-activity relationship, Ukr. Biokhim. Zh. 80 (2008) 140–147.
[15] L.S. Thomashow, D.M. Weller, Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites, Plant Microbe Interact. 1 (1996) 187–236.
[16] T. Morohoshi, W.Z. Wang, T. Suto, Y. Saito, S. Ito, N. Someya, et al., Phenazine antibiotic production and antifungal activity are regulated by multiple quorum-sensing systems in *Pseudomonas chlororaphis* subsp. *aurantiaca* StFRB508, J. Biosci. Bioeng. 116 (2013) 580–584.
[17] L.S. Thomashow, D.M. Weller, Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*, J. Bacteriol. 170 (1988) 3499–3508.
[18] C.E.J. Vanrensburg, G.K. Joone, J.F. Osullivan, R. Anderson, Antimicrobial activities of clofazimine and B669 are mediated by lysophospholipids, Antimicrob. Agents Chemother. 36 (1992) 2729–2735.

[19] M.C. Cholo, H.I. Boshoff, H.C. Steel, R. Cockeran, N.M. Matlola, K.J. Downing, et al., Effects of clofazimine on potassium uptake by a Trk-deletion mutant of *Mycobacterium tuberculosis*, J. Antimicrob. Chemother. 57 (2006) 79–84.
[20] M.C. Bopape, H.C. Steel, R. Cockeran, N.M. Matlola, P.B. Fourie, R. Anderson, Antimicrobial activity of clofazimine is not dependent on mycobacterial C-type phospholipases, J. Antimicrob. Chemother. 53 (2004) 971–974.
[21] A. Terenzi, L. Tomasello, A. Spinello, G. Bruno, C. Giordano, G. Barone, Dipyrido[3,2-a:2′,3′-c]phenazine)(glycinato)copper(II) perchlorate: a novel DNA-intercalator with anti-proliferative activity against thyroid cancer cell lines, J. Inorg. Biochem. 117 (2012) 103–110.
[22] N.E. Morrison, G.M. Marley, The mode of action of clofazimine DNA binding studies, Int. J. Lepr. Other Mycobact. Dis. 44 (1976) 133–134.
[23] N.E. Morrison, G.M. Marley, Clofazimine binding studies with deoxyribonucleic acid, Int. J. Lepr. Other Mycobact. Dis. 44 (1976) 475–481.
[24] A.S. Hussain, J. Bozinovski, D.H. Maurice, B.E. McLaughlin, G.S. Marks, J.F. Brien, et al., Inhibition of the action of nitric oxide prodrugs by pyocyanin: mechanistic studies, Can. J. Physiol. Pharmacol. 75 (1997) 398–406.
[25] D.K. Morales, N.J. Jacobs, S. Rajamani, M. Krishnamurthy, J.R. Cubillos-Ruiz, D.A. Hogan, Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms, Mol. Microbiol. 78 (2010) 1379–1392.
[26] T. Yano, S. Kassovska-Bratinova, J.S. Teh, J. Winkler, K. Sullivan, A. Isaacs, et al., Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species, J. Biol. Chem. 286 (2011) 10276–10287.
[27] Y.P. Hou, Z.T. Zheng, S. Xu, C.J. Chen, M.G. Zhou, Proteomic analysis of *Fusarium graminearum* treated by the fungicide JS399-19, Pestic. Biochem. Physiol. 107 (2013) 86–92.
[28] Z.M. Yang, L.K. Jiang, Biochemistry, Higher Education Press, Beijing, 2005.
[29] K. Schauer, D.A. Rodionov, H. de Reuse, New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'? Trends Biochem. Sci. 33 (2008) 330–338.
[30] X. Zeng, F. Xu, J. Lin, Specific TonB-ExbB-ExbD energy transduction systems required for ferric enterobactin acquisition in *Campylobacter*, FEMS Microbiol. Lett. 347 (2013) 83–91.
[31] D.L. Zimbler, B.A. Arivett, A.C. Beckett, S.M. Menke, L.A. Actis, Functional features of TonB energy transduction systems of *Acinetobacter baumannii*, Infect. Immun. 81 (2013) 3382–3394.
[32] K.K. Brinkman, R.A. Larsen, Interactions of the energy transducer TonB with noncognate energy-harvesting complexes, J. Bacteriol. 190 (2008) 421–427.
[33] K. Postle, R.A. Larsen, TonB-dependent energy transduction between outer and cytoplasmic membranes, Biometals 20 (2007) 453–465.
[34] J. Ghosh, K. Postle, Disulphide trapping of an *in vivo* energy-dependent conformation of *Escherichia coli* TonB protein, Mol. Microbiol. 55 (2005) 276–288.
[35] K.G. Held, K. Postle, ExbB and ExbD do not function independently in TonB-dependent energy transduction, J. Bacteriol. 184 (2002) 5170–5173.
[36] C. Miyaguchi, S. Muranaka, T. Kanno, H. Fujita, J. Akiyama, T. Yoshioka, et al., 17beta-estradiol suppresses ROS-induced apoptosis of CHO cells through inhibition of lipid peroxidation-coupled membrane permeability transition, Physiol. Chem. Phys. Med. NMR 36 (2004) 21–35.