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EXPERIMENTAL ARTICLES

Activation of the Antioxidant Complex in *Pseudomonas aurantiaca*—Producer of Phenazine Antibiotics

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Abstract—Two *Pseudomonas aurantiaca* mutant strains overproducing phenazine antibiotics (synthesis levels of 210 and 410 mg/l, respectively) along with wild-type bacteria (production level of 71–75 mg/l) and a phz$^-$ mutant not producing phenazines were used to study the changes in the activity of the antioxidant complex components, that is, catalase, superoxide dismutase (SOD), glutathione reductase, and NADH oxidase; glutathione concentration (in both reduced and oxidized forms); and activity of acyl-CoA synthetase, the key enzyme of cell metabolism.

Bacterial producers were found to respond to an increase in intra- and extracellular phenazines by induction of catalase, SOD, glutathione reductase, and glutathione synthesis. However, while in the case of catalase and glutathione reductase this trend was observed in all the strains under study, the activity of SOD at a high level of phenazine synthesis (in particular, 410 mg/l) decreased somewhat, probably due to high its sensitivity to high concentrations of H_2O_2 generated by phenazines. Decrease in SOD activity was compensated by increase in the synthesis rates of glutathione and glutathione reductase. NADH oxidase was shown to be practically uninvolved in formation of *P. aurantiaca* response toward phenazine accumulation, and acyl-CoA synthetase activity was found to decrease.

Key words: phenazine antibiotics, catalase, superoxide dismutase, glutathione reductase, NADH oxidase, acyl-CoA synthetase.

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Phenazine antibiotics are low-molecular weight heterocyclic nitrogen-containing compounds synthesized in the course of the aromatic pathway reactions. All the compounds of the group are based on phenazine-1-carboxylate (PCA), with phenazine, 2-oxyphenazine-1-carboxylate, pyocyanin, oxychlororaphin, and hemipyocyanin (1-hydroxyphenazine) being derivatives of it (Fig. 1) [1, 2].

Compounds of the phenazine group exhibit high antimicrobial activity toward fungi and gram-negative and gram-positive bacteria [3]. Their wide range of activity is due to the mechanism of action. The compounds penetrate easily inside bacterial cells, where they become involved in oxidation–reduction reactions by accepting electrons from glutathione and NADH accompanied by formation of relatively stable anions which, in turn, activate the production of reactive oxygen species ($O_2^{\cdot -}$, OH^-), inducing oxidative stress followed by death of the microorganisms [4, 5]. In addition, phenazine antibiotics were shown to be specific inhibitors of acyl CoA-synthetase [6], an enzyme catalyzing formation of active fatty acid intermediates in the process of cellular lipid synthesis that are important regulators of metabolism and gene expression [7].

Meanwhile, not all microorganisms are sensitive to phenazine antibiotics. First of all, bacteria capable of phenazine synthesis, that is, representatives of the genera *Pseudomonas, Burkholderia, Brevibacterium, Bacillus, Streptomyces*, and *Methanosarcina*, are resistant [8]. In particular, *P. aeruginosa*, bacteria producing pyocyanin, were shown to be pyocyanin-resistant, maintaining viability even upon exogenous introduction of excess amounts of pyocyanin (for example, at a concentration of 0.1 mM, or 21 mg/l) to culture medium [9]. However, the reasons for the resistance of *P. aeruginosa* to excess pyocyanin were not studied by the authors. Wild-type bacteria were only demon-

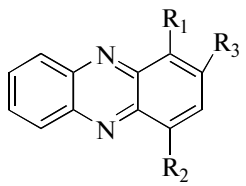

Fig. 1. Structure of phenazine antibiotics. R_1, R_2, $R_3 = 0$, phenazine; R_1 = COOH, phenazine -1-carboxylate; OH, 1-hydroxyphenazine; $CONH_2$, oxychlororaphin; R_1 = O^-, R_2 = CH_3, pyocyanin; R_1 = COOH, R_3 = OH, 2-oxyphenazine-1-carboxylate; R_1 = COOH, R_2 = CH_3, 5-methylphenazine-1-carboxylate.

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strated to increase pyocyanin production 11-fold and the activity of two enzymes, catalase and SOD, by 4.8 and 5 times, respectively, under limitations in phosphorus availability. The leading role in the organisms' protection against oxidative stress is known to belong to the antioxidant complex comprising catalase, superoxide dismutase (SOD), NADH oxidase, and glutathione and the related enzyme complex responsible for glutathione conversion from the oxidized to the reduced form [10]. By the example of gram-positive bacteria *Bacillus* sp. [11], acyl-CoA synthetase was demonstrated to participate in the development of resistance to phenazines.

Therefore, the mechanisms of bacterial resistance to phenazines have not been studied in detail to date. Moreover, there are absolutely no data concerning bacteria capable of overproduction of phenazine antibiotics, despite the fact that such strains have been obtained for a number of bacteria and are of great interest due to the possibility of their use as new biopesticides.

The aim of this work was to study the mechanisms of *P. aurantiaca* resistance to phenazines produced by the bacteria themselves.

MATERIALS AND METHODS

In the work, *Pseudomonas aurantiaca* strain B-162 from the collection of the Department of Genetics of Belarus State University (collection record number KMBU-162) was used. Its regulatory mutants capable of phenazine overproduction [12] and phz$^-$ mutants that have lost the ability to synthesize the compounds were obtained by transposone mutagenesis.

Bacteria were grown at 28°C in a liquid nutrient medium with or without aeration depending on the purposes of the experiment, as well as on agarized medium of the same composition. To study phenazine production, bacteria were cultured in a special PCA medium [13] at 28°C for 5 days. Transposone mutagenesis was performed according to the procedure proposed earlier [14] using *E. coli* strain S17/1(pro$^-$; thi$^-$) pUT(Ap^R::miniTn5(Sm^R) as donor and *P. aurantiaca* as recipients.

The numbers of viable bacteria were determined by serial dilutions. Cellular extract was obtained by ultrasonic treatment of cellular suspensions (30 kHz, 3 × 15 s) at 4°C in 0.015 M phosphate buffer.

Isolation and quantitation of phenazine antibiotics was performed according to a procedure described earlier [13].

SOD activity was determined based on spectrophotometric detection of the changes in quercetin concentration with time [15]. Catalase activity was determined according to the known technique proposed by Aebi [16]. NADH oxidase activity was determined spectrophotometry by a decrease in NADH concentration [17]. Oxidized and reduced forms of glutathione were registered using a technique developed by Senft et al. [18]. Glutathione reductase activity was registered spectrophotometrically as proposed in [19]. Activity of SOD was expressed as SU/mg protein (1 SU corresponds to 50% inhibition of quercetin degradation per mg protein); of catalase, as mmol/(min mg protein); and of NADH oxidase and glutathione reductase, as μmol/(min mg protein).

Spectrophotometric analyses were performed on a Cary 50 scan spectrophotometer (Varian, Australia). Protein determination was carried out according to the Bradford technique [20].

Acyl-CoA synthetase activity was evaluated by change in oleoyl-CoA concentration in the reaction mixture assessed in an LCMS-QP8000α liquid chromatograph coupled to a mass spectrometry detector upon elution on a Restec Allure C18 (150 ± 4.6 mm, 60 Å) reverse-phase column. Absorbance spectra of the flow were registered with an SPD-M10Avp photodiode matrix detector at 254 nm [21].

RESULTS AND DISCUSSION

Earlier, mutant strains B-162/55 and B-162/255 were obtained by a series of consequent mutageneses with selection of the mutants resistant to the toxic analogues performed on the rhizosphere bacteria *P. aurantiaca* B-162 (phenazine antibiotics production 71–75 mg/l), which produced three to six times more antibiotics (210 and 410 mg/l, respectively) [12]. Moreover, phenazine was found to be the major component of *P. aurantiaca* B-162 antibiotics, while 1-oxyphenazine was the minor one [22].

The viability of the mutant bacteria B-162/55 and B-162/255 under conditions of phenazine overproduction (upon cultivation on PCA media) practically did not change and was comparable to that of the wild-type bacteria (Fig. 2a). It should be noted that the rate of synthesis of phenazine antibiotic observed in the mutants was relatively high, 10–20 times that of the relevant *P. chlororaphis* and *P. aeruginosa* strains [23].

The resistance of *P. aurantiaca* antibiotic-producer strains to high concentrations of self-produced phenazines was assumed to be connected with a powerful antioxidant system enabling not only normal cell viability under these conditions, but also the capacity for antibiotics overproduction.

During the first stage of the work, comparative analysis of the activity profiles of catalase, one of the major enzymes of the antioxidant complex, was performed for the phenazine-producing strains, as well as for wild-type bacteria and B-162phz$^-$ mutants completely lacking the capacity for phenazine synthesis. The specific activity of catalase in mutants B-162/55 and B-162/255 was found to increase dramatically, by factors of 4.4 and 10.8, respectively, compared to the wild-type bacteria, and by factors of 6.7 and 16.4, compared to the B-162phz$^-$ variant (Fig. 2b).

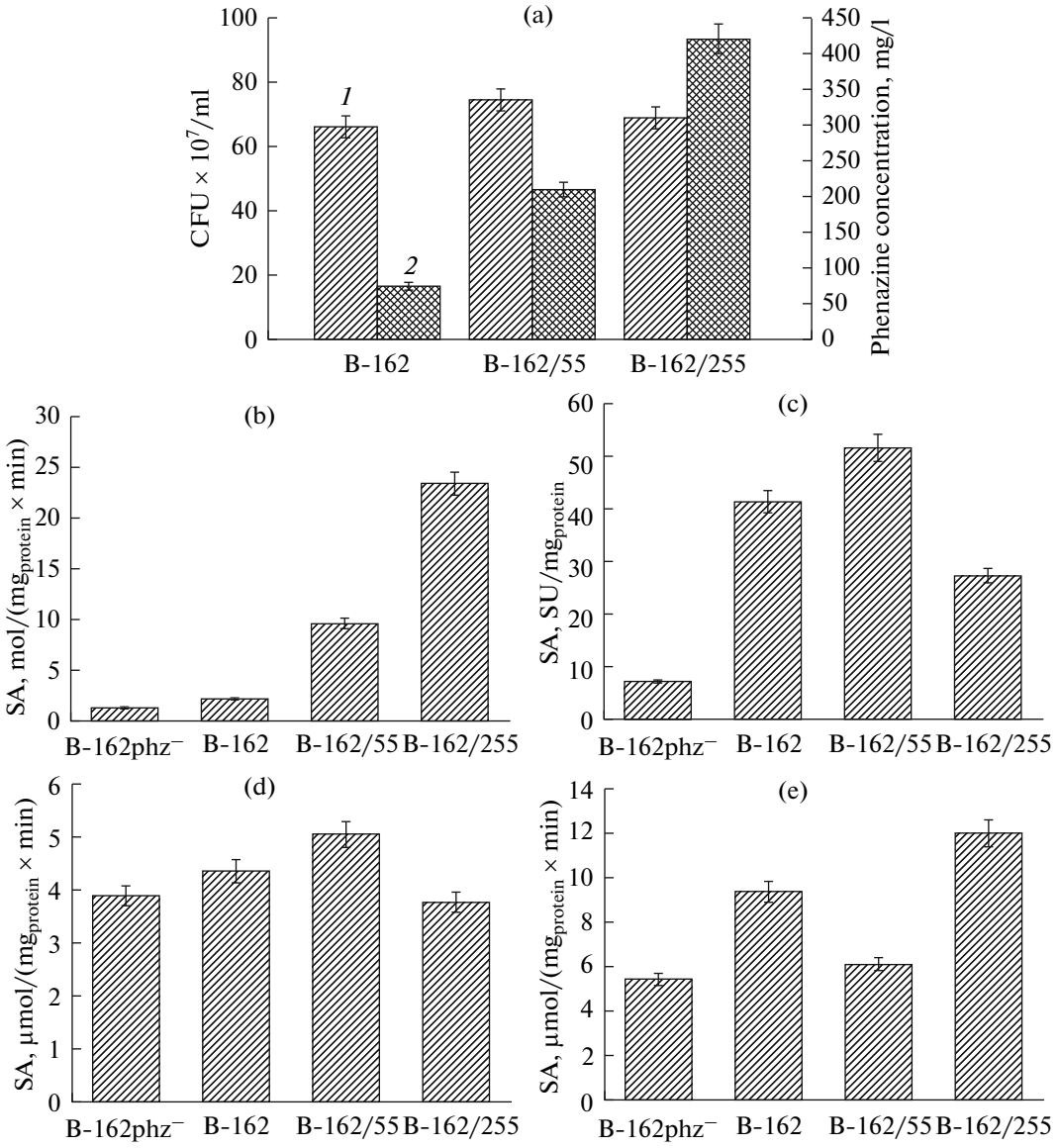

Fig. 2. Phenazine production and activity of the enzymes of the antioxidant complex in *P. aurantiaca* B-162 and its mutants: (a) CFU × 10^7/ml (*1*), phenazine concentration, mg/l (*2*). Specific activity of catalase (b), SOD (c), NADH oxidase (d), and glutathione reductase (e). SA, specific activity.

In the phz⁻ mutants, the specific activity of catalase was low, not exceeding 1.4 mmol/(min mg protein), while for the strain with the highest phenazine production level it was to 23.3 mmol/(min mg protein). In addition, the values of specific activity of the enzyme in mutant bacteria were found to correlate with the level of antibiotics production (Figs. 2a and 2b).

The data obtained may be explained on the basis of the modern concepts of the mechanisms of phenazine activity [10], according to which phenazines leave bacterial cells easily and generate ($O_2^{\cdot-}$). Due to the high reactivity, this oxygen form immediately gets involved in spontaneous transformations leading to hydrogen peroxide formation. It is most likely that H_2O_2 is the compound formed in *P. aurantiaca* upon bulk production of phenazines, which explains the significant increase in catalase activity in phenazine overproducing strains B-162/55 and B-162/255. This, in turn, evidences the induction of catalase synthesis in these strains. Earlier, using the example of *Lactobacillus sakei*, catalase synthesis was demonstrated to be inducible [24].

Moreover, SOD activity profiles were studied in the phenazine producers B-162/55 and B-162/255 in comparison to the wild type bacteria and the B-162phz⁻ mutant. The levels of specific activity of

Different glutathione forms content in *P. aurantiaca* B-162 and its mutants

Strains	$C_{ox\ glutathione}$, nM	$C_{red\ glutathione}$, nM	$\Sigma C_{glutathione}$, nM	$C_{ox} : C_{red}$
B-162phz$^-$	8 ± 1.5	11 ± 1.2	17	1 : 1.38
B-162	18 ± 3.0	28 ± 2.1	46	1 : 1.56
B-162/55	33 ± 1.2	43 ± 2.7	83	1 : 1.40
B-162/255	55 ± 4.5	88 ± 2.4	143	1 : 1.60

this enzyme were expected to increase along with the increase of phenazine production. Indeed, the trend was observed in the wild-type bacteria (phenazine production 71–75 mg/l) and one of the overproducing strains, B-162/55 (210 mg/l phenazine production); the enzyme specific activity increased by factors of 5.9 and 7.4, respectively. Unexpectedly, the value decreased in the case of another producer strain, B-162/255, with phenazine production up to 410 mg/l (Fig. 2c). It may be assumed that massive generation of H_2O_2 (or $(O_2^{\cdot-})$ in the presence of high phenazine concentrations inhibits SOD activity. This hypothesis is supported by the observation that one of the most common SOD isoforms in microorganisms, Fe-SOD [25], is sensitive to high hydrogen peroxide concentrations and is vulnerable to inhibition in the presence of H_2O_2 in concentrations above a certain threshold level [11, 26].

A decrease in SOD activity at high levels of phenazine production may be compensated for by another alternative mechanism for elimination of reactive oxygen species, that is, by increased levels of intracellular glutathione, which, according to a number of authors, may compensate low SOD activity. Earlier, using the example of *Lactococcus lactis*, the latter was demonstrated to be able to compensate low SOD activity under conditions of oxidative stress induced by hydrogen peroxide [11].

Glutathione is known to be the major factor protecting the organism against reactive oxygen species. In gram-negative bacteria, including *Pseudomonas*, glutathione was detected in relatively high concentrations. Being part of the enzyme-controlled redox system, glutathione performs a protective function [27]. Earlier, it was demonstrated that, in some bacteria (*Haemophilus infuenzae* and *Rhizobium tropici*), intracellular glutathione concentration may increase in the presence of H_2O_2 in a concentration-dependent manner [28, 29]. Based on this, we studied glutathione content in the strains of interest and analyzed the ratio between its oxidized and reduced forms.

It was demonstrated that the initial level of glutathione was relatively high in the cells under study and independent of the phenazine synthesis rate (in particular, 17 nM for the mutant B-162phz$^-$ not producing phenazines). In addition, glutathione accumulation in wild-type cells and producer strains was found to correlate with phenazine synthesis. In particular, an increase in total glutathione concentration by factors of 2.7, 4.9, and 8.4 compared to strain B-162phz$^-$ was registered in bacteria strains B-162, B-162/55, and B-162/255, respectively (table). The data obtained demonstrate induction of glutathione synthesis in phenazine-producing strains, along with other adaptive responses providing protection against self-produced toxic compounds. Increase in glutathione concentration may be accomplished through activation of γ-glutamyl synthase along with the positive transcription regulation of the genes encoding the enzyme heavy chain in the presence of H_2O_2 [30]. Increase in total glutathione concentration in strain B-162/255 may also evidence the cells' compensatory response toward a decrease in SOD activity.

We also investigated the synthesis rates for glutathione reductase, an enzyme transforming oxidized glutathione into its reduced form. According to the data presented in Fig. 2e, compared to strain B-162phz$^-$ (incapable of phenazine synthesis), an increase in the specific activity of the enzyme by factors of 1.7 and 2.2 was observed in strains B-162 and B-162/255, respectively. This may explain some increase in the concentration of reduced glutathione in the bacteria (table). As for the strain B-162/55, its glutathione reductase activity did not differ from the control and the ratio between the oxidized and reduced glutathione forms was close to that of B-162phz$^-$. SOD activation and increase in glutathione synthesis rates were probably the main protective mechanisms for this strain.

At the following stage, we analyzed the activity of NADH oxidase, which is known to participate in protection against oxidative stress, especially in facultative anaerobic microorganisms, including *Lactococcus lactis* [11]. Experiments were therefore set up to study the enzyme levels in all the strains under study, that is B-162, B-162/55, B-162/255, and B-162phz$^-$. NADH oxidase activity of the strains was found to be practically independent of the levels of phenazine

antibiotics production. Apparently, this mechanism of protection against oxidative stress is not typical for *P. aurantiaca* and does not participate in formation of the cellular response toward increased phenazine synthesis rates (Fig. 2d).

Since phenazine antibiotics are known to be specific inhibitors of acyl-CoA synthetase activity and decrease in the sensitivity of the latter to phenazines is regarded as one of the mechanisms of bacterial resistance to such compounds [10], we considered it of interest to study the levels of acyl-CoA synthetase specific activity in *P. aurantiaca* strains under study in comparison to wild-type bacteria and the B-162phz$^-$ variant (Fig. 3).

Increased phenazine production levels caused a decrease in the enzyme specific activity in all strains starting from B-162phz$^-$ to B-162/255 which was characterized by the value of acyl-CoA synthetase activity 3.2 times lower than the control one (strain B-162phz$^-$). It may be concluded that in *P. aurantiaca* B-162, acyl-CoA synthetase is one of the sensitive targets for phenazine antibiotics. However, the decrease of its activity apparently is not critical for *P. aurantiaca* and even the low enzyme activity of 5 $\mu g_{oleoylCoA}/(min \times mg_{protein})$ observed in B-162/255 cells allowed normal cell functioning and phenazine synthesis in rather high concentrations.

Therefore, the data obtained demonstrate that high phenazine synthesis rates in *P. aurantiaca* producer strains correlate to a decrease in acyl-CoA synthetase specific activity and increase in the activity of some components of the antioxidant complex. The major contribution to the process is provided by catalase, with a 16.4-fold increase in specific activity, directly depending on the phenazine synthesis rate. The picture is similar in the case of total glutathione concentrations. Meanwhile, it was found that NADH oxidase did not participate in protective response to phenazine activity, since its specific activity practically did not change in the strains with increased productivity. Interestingly, a mutual compensating effect was observed for SOD and glutathione reductase. For example, an increase in SOD activity level resulted in a decrease in glutathione reductase activity in strain B-162/55, while, on the contrary, SOD activity decreased with increasing glutathione reductase activity in strain B-162/255.

Analysis of our results and the previously known data on catalase activation under the effect of hydrogen peroxide [24], inhibition of SOD activity upon the achievement of a threshold H_2O_2 concentration [26], and increase in glutathione synthesis [30] allows one to assume that it is hydrogen peroxide that induces cellular response to the toxic activity of phenazines in *P. aurantiaca* producers. In the case of acyl-CoA synthetase, the most probable reason for the decrease of its activity in phenazine-producing strains is enzyme sensitivity to phenazines similar to what had been previously demonstrated by the example of *Pseudomonas* sp. [6].

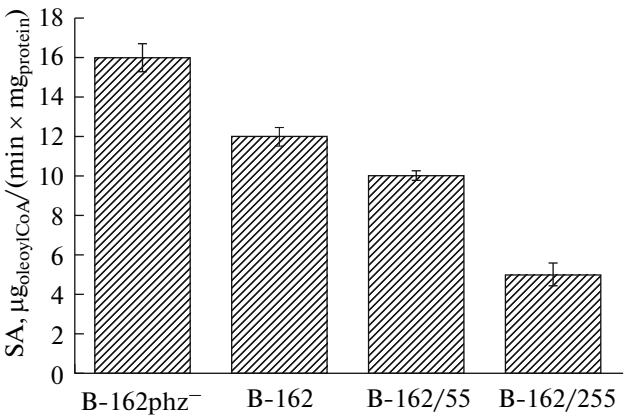

Fig. 3. Acyl-CoA synthetase specific activity in *P. aurantiaca* B-162 and its mutants. The results of three experiments are presented. Specific activity was calculated using the Labsolutions software package. SA, specific activity.

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