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EXPERIMENTAL ARTICLES

Obtaining *Pseudomonas aurantiaca* Strains Capable of Overproduction of Phenazine Antibiotics

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Abstract—N-methyl-N'-nitro-N-nitrosoguanidine (NG)-induced mutagenesis with subsequent selection for resistance to toxic amino acid analogues (azaserine, *m*-fluoro-DL-phenylalanine, and 6-diazo–5-oxo-L-norleucine) was applied to *Pseudomonas aurantiaca* B-162. The resulting strains produced phenazine antibiotics three times more efficiently than the wild type strain and ten times more efficiently than the known pseudomonad strains. Overproduction of phenazine antibiotics was shown to result either from deregulation of 3-deoxy-D-arabinohepulosonate-7-phosphate synthase (DAHP synthase), the key enzyme of the aromatic pathway (removal of inhibition by phenylalanine, tyrosine, and phenazine), or overproduction of N-hexanoyl homoserine lactone, the regulatory molecules of positive control of cellular metabolism (QS systems).

Key words: Pseudomonas, phenazine antibiotics, producers, mutants.

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Over 20 *Pseudomonas* species are known to synthesize antibiotics and antibiotic-like compounds; these bacteria belong primarily to the soil and rhizosphere pseudomonads of the "fluorescent group" (*Pseudomonas fluorescens*, *P. aureofaciens*, *P. chlororaphis*, *P. putida*, etc.). More than 100 aromatic antibiotics have been revealed in pseudomonads; their importance is due to their activity against a number of animal and plant pathogens [1–4]. Phenazine type antibiotics, nitrogen-containing aromatic compounds, are especially active against lower fungi and most gram-positive and gram-negative phytopathogenic bacteria. High antimicrobial activity of phenazine compounds results from their capacity to block oxidation–reduction reactions and thus disrupt cellular respiration, as well as induce excessive accumulation of oxidizing radicals in sensitive cells [7]. Bacterial producers of antibiotics use highly active enzymes (catalase and peroxidase) to inactivate the products of peroxide oxidation; their cells are therefore not sensitive to phenazine compounds.

Phenazine antibiotics are synthesized via the aromatic pathway. Condensation of erythrose-4-phosphate and phosphoenolpyruvate is the initial reaction, catalyzed by 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP synthase, E.C. 4.1.2.15). Seven common reactions follow; the subsequent synthesis of phenazine compounds follows a specific pathway.

The aromatic pathway in pseudomonads is regulated at the DAHP synthase level by allosteric inhibition and repression of its synthesis [8, 9]. Among the mechanisms of regulation of specific pathways of aromatic antibiotics synthesis, the best known are the transcriptional, involving σ factors and the quorum-sensing system (QS system) requiring N-acyl-homoserine lactone (N-AHL), and the posttranscriptional, involving the GacS/GacA two-component system [10, 11]. Both mechanisms participate in the positive regulation of bacterial secondary metabolism. Such mechanisms of negative control as retro inhibition and repression are probably not features of the phenazine pathway. The regulatory mutations enhancing the aromatic pathway and optimization of the positive control systems are therefore the major approaches to increased antibiotic synthesis.

The phenazine antibiotics complex of *P. aurantiaca* B-162 consists of 1-oxyphenazine, phenazine, and phenazine-1,6 dicarboxylate (their common precursor). Antibiotic yield under optimized conditions of cultivation time, temperature, and aeration was 71.11 ± 2.72 mg/l [12].

The goal of the present work was to obtain and characterize the *P. aurantiaca* B-162 regulatory mutants capable of overproduction of phenazine antibiotics.

MATERIALS AND METHODS

Strain *Pseudomonas aurantiaca* B-162 from the collection of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences was used in the work. Bacteria were grown for two days at 28°C on a rotary shaker (180 rpm) in M9 liquid medium

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[13]. To obtain the mutants, the cells were treated for 40 min with a 200 μg/ml solution of N-methyl-N'-nitro-N-nitrosoguanidine (NG) in 0.1 M citrate buffer (pH 5.5) at 28°C. The cells were washed by centrifugation (8000 *g*), plated on M9 agarized media supplemented with a necessary concentration of one of the analogues (*m*-fluoro-DL-phenylalanine, azaserine, or 6-diazo-5-oxo-L-norleucine), and incubated for five days. The level of production of phenazine compounds by the analogue-resistant clones was then determined. In order to determine the amount of phenazine antibiotics, medium of the following composition (g/l) was used: peptone (Difco), 20; glycerol, 10; NaCl, 5; KNO_3, l; pH 7.2.

The phenazine antibiotics were determined as described by Levitch [14]. Antibiotic concentrations were determined spectrophotometrically at 369 nm.

DAHP synthase activity was determined spectrophotometrically at 549 nm according to the method described in [15] by means of a Cary WinUV spectrophotometer.

N-AHL was isolated according to the procedure described by McClean et al. [16]; the components were separated of Silica Gel 60 F_{254} TLC plates (Merck, Germany). To identify the components, *Chromobacterium violaceum* CV026 was used, which synthesizes a blue violacein pigment in the presence of N-acyl-homoserine lactone.

RESULTS AND DISCUSSION

In a preliminary series of experiments, the capability of *P. aurantiaca* B-162 to grow on media containing the toxic analogues of the aromatic pathway intermediates (α-methyl-DL-phenylalanine, *p*-fluoro-DL-phenylalanine, *m*-fluoro-DL-phenylalanine, 2-fluoro-DL-phenylalanine, *m*-fluoro-DL-tyrosine, 5-methyl-DL-tryptophan, 1-methyl-DL-tryptophan, 5-fluoro-DL-tryptophan, azaserine, and 6-diazo-5-oxo-L-norleucine) was tested. Only some of these compounds (azaserine, 6-diazo-5-oxo-L-norleucine, 5-fluoro-DL-tryptophan, and *m*-fluoro-DL-phenylalanine) were found to impede the growth of *P. aurantiaca* B-162. NG was then used to obtain over 500 *P. aurantiaca* B-162 mutants resistant to azaserine, *m*-fluoro-DL-phenylalanine, and 6-diazo-5-oxo-L-norleucine. From these isolates, the mutants were selected capable of overproduction of phenazine antibiotics (Table 1).

Table 1. Synthesis of phenazine antibiotics by *P. aurantiaca* B-162 regulatory mutants

P. aurantiaca strain	Toxic analogues used for mutant selection	Phenazine production, mg/l
B-162 (control)	–	71.11 ± 2.72
B-162/57	*m*-Fluoro-DL-phenylalanine	167.73 ± 1.14
B-162/115	Azaserine	141.40 ± 2.32
B-162/272	Azaserine	180.02 ± 2.64
B-162/274	6-Diazo-5-oxo-L-norleucine	183.61 ± 2.12
B-162/351	Azaserine	159.14 ± 1.53
B-162/352	Azaserine	160.35 ± 2.44
B-162/490	6-Diazo-5-oxo-L-norleucine	181.30 ± 2.73
B-162/492	Azaserine	147.23 ± 1.30
B-162/494	Azaserine	136.31 ± 1.22
B-162/495	6-Diazo-5-oxo-L-norleucine	143.80 ± 2.00
B-162/497	Azaserine	193.71 ± 2.22
B-162/498	6-Diazo-5-oxo-L-norleucine	205.32 ± 1.91
B-162/499	Azaserine	158.53 ± 1.82

These results demonstrate that the analogue-resistant strains synthesize two to three times more antibiotics than wild type bacteria. The productivity of the strains thus obtained was stably high and did not decrease in the course of 19-month observations. A threefold increase in phenazine antibiotics synthesis has been previously reported for *P. fluorescens* after cloning of the *phz* operon genes within the pUTKm-*phz* plasmid [17]. Our results demonstrate an easier method to achieve a similar effect, by selection of mutants resistant to the toxic analogues of the aromatic pathway metabolites.

High levels of synthesis of the terminal products of the aromatic pathway are known to be determined by both high specific activity of the key enzymes of this pathway and by deregulation of their activity. The regulatory mutants resistant to the toxic analogues of the aromatic pathway metabolites and capable of overproduction of the aromatic pathway antibiotics were tested for the removal of inhibition DAHP synthase, the key enzyme of the aromatic pathway, by aromatic amino acids and antibiotics. The regulation of DAHP synthase activity was studied in a partially purified extract of *P. aurantiaca* B-162 cells; inhibition by tyrosine and phenylalanine was shown. A 50% inhibition of DAHP synthase occurred at tyrosine and phenylalanine concentrations of 5.5×10^{-6} and 1.3×10^{-5} M, respectively (Fig. 1). At an inhibitor concentration of 10^{-3} M, the highest level of inhibition was achieved (78 and 61% for tyrosine and phenylalanine, respectively). The sigmoid shape of the graph of the enzymatic activity as a function of the inhibitor concentration indicates an allosteric mechanism of the *P. aurantiaca* B-162 DAHP synthase by phenylalanine and tyrosine; this is the case for all the bacteria studied in this respect [18].

The effect of phenazine on DAHP synthase activity in the bacteria under study was determined at varied substrate concentrations; the results were presented as Lineweaver–Burk plots. Phenazine was shown to cause uncompetitive inhibition of this enzyme (Fig. 2). Anal-

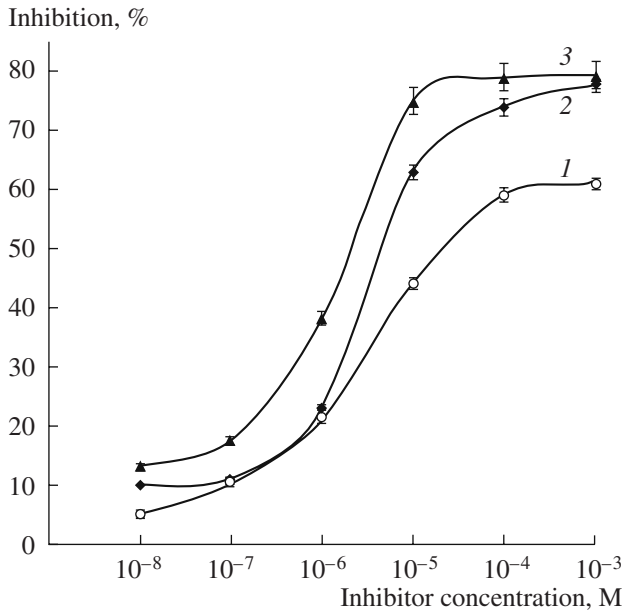

Fig. 1. Inhibition of DAHP synthase activity in *P. aurantiaca* B-162 by aromatic amino acids: tyrosine (*1*), phenylalanine (*2*), and a mixture of aromatic amino acids (*3*).

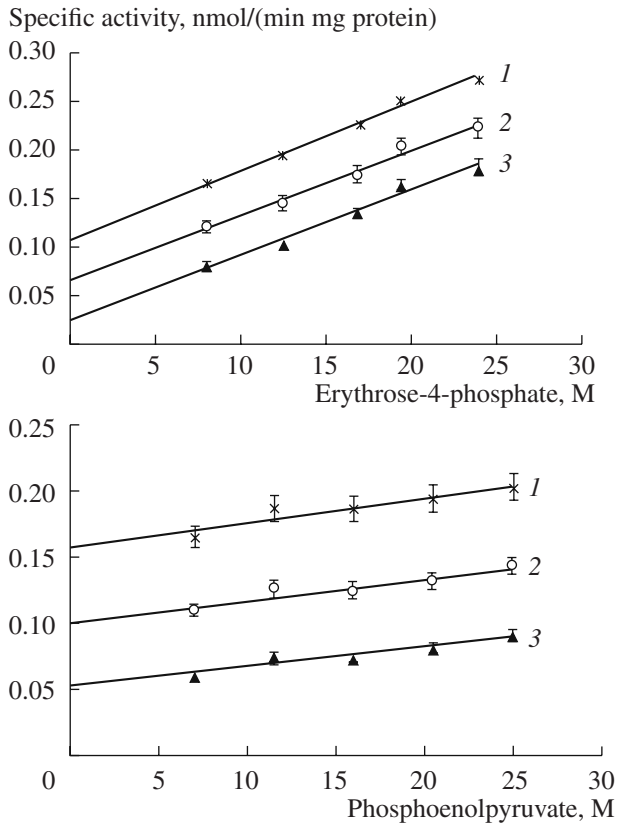

Fig. 2. Inhibition of DAHP synthase activity in *P. aurantiaca* B-162 by phenazine depending on erythrose-4-phosphate and phosphoenolpyruvate concentrations: 0.1 M phenazine (*1*); 0.01 M phenazine (*2*); and without inhibitors (*3*).

ysis of the regulation of DAHP synthase activity in *P. aurantiaca* B-162 regulatory mutants revealed that the enzyme was less sensitive to amino acids and phenazine antibiotics. The specific activity of DAHP synthase was the same in the mutants and in the original strain (Table 2).

Three groups of *P. aurantiaca* B-162 regulatory mutants can be defined depending on the character of the inhibition of DAHP synthase activity.

Group 1: strains *P. aurantiaca* B-162/351, B-162/499, and B-162/494; unlike the original strain, their DAHP synthase is less sensitive to tyrosine and phenazine inhibition.

Group 2: strains *P. aurantiaca* B-162/492, B-162/495, and B-162/115; their DAHP synthase is less sensitive to phenylalanine and tyrosine inhibition.

Group 3: strains *P. aurantiaca* B-162/352, B-162/272, B-162/490, B-162/274, B-162/497, and B-162/498; their DAHP synthase is less sensitive to phenylalanine and phenazine inhibition (Table 2).

In the case of allosteric inhibition, as in the interaction between DAHP synthase and aromatic amino acids, the effector molecules are known to attach to the enzyme outside its active center; the enzyme conformation is therefore changed. In *P. aurantiaca* B-162/492, B-162/495 and B-162/115 (group 2) the mutations resulting in DAHP synthase desensibilization are probably in the regions of the genes responsible for the amino acids corresponding to the isoenzymes' allosteric center. In *P. aurantiaca* B-162 strains belonging to groups 1 and 3, NG-induced mutations probably resulted in the inability of DAHP synthase [tyr] to interact with tyrosine (group 1) and of DAHP synthase [phe] with phenylalanine (group 3). Since phenazine causes noncompetitive inhibition of the enzyme, the loss of DAHP synthase sensitivity to inhibition by phenazine resulted from mutations in the regions responsible for binding of phenazine antibiotics with specific sites after formation of the enzyme–substrate complex. Analysis of these results suggests the conclusion that the highest level of phenazine antibiotics production (160–205 mg/l) occurs in the regulatory mutants with DAHP synthase resistant to inhibition by phenylalanine and phenazine.

In one of the mutants (*P. aurantiaca* B-162/57), overproduction of phenazine antibiotics (167.7 ± 1.1 mg/l) was not related to DAHP synthase deregulation; its activity was inhibited by phenylalanine, tyrosine, and phenazine in a manner similar to the initial strain *P. aurantiaca* B-162 (Table 2). The reason for this interesting phenomenon was investigated. Since the QS system is known to be responsible for the regulation of the synthesis of phenazine compounds, overproduction in the *P. aurantiaca* B-162/57 mutant was assumed to be the result of an elevated N-AHL level. TLC of the *P. aurantiaca* B-162 culture liquid revealed a metabo-

Table 2. Inhibition of DAHP synthase activity in *P. aurantiaca* regulatory mutants by the end products of the aromatic pathway

P. aurantiaca strain	Group no.	Specific activity of DAHP synthase, nmol/(min mg protein)	Inhibition, %		
			phenylalanine	tyrosine	phenazine
B-162	–	14.72 ± 0.45	60.71	78.35	39.91
B-162/351	1	14.57 ± 0.27	55.12	20.38	10.39
B-162/494	1	15.00 ± 0.13	56.72	47.21	27.38
B-162/499	1	14.82 ± 0.13	55.05	15.27	8.12
B-162/492	2	15.29 ± 0.31	4.04	10.13	38.62
B-162/495	2	15.15 ± 0.41	40.01	20.84	37.74
B-162/115	2	15.09 ± 0.45	42.11	3.12	40.73
B-162/352	3	15.26 ± 0.15	4.23	76.13	5.50
B-162/272	3	14.51 ± 0.67	39.70	73.38	10.52
B-162/490	3	14.84 ± 0.42	14.91	74.30	4.81
B-162/274	3	14.88 ± 0.73	13.14	67.61	8.84
B-162/497	3	15.07 ± 0.18	2.17	79.10	5.85
B-162/498	3	14.93 ± 0.57	5.46	75.92	1.17
B-162/57	–	14.97 ± 0.51	57.24	73.54	39.30

Note: Group 1, strains with DAHP synthase less sensitive to inhibition by tyrosine and phenylalanine; group 2, by phenylalanine and tyrosine; group 3, by phenylalanine and phenazine.

lite with an R_f value of 0.68 (Fig. 3). The compound was identified as N-hexanoyl homoserine lactone (N-HHL) by means of the N-AHL test with the indicator strain *C. violaceum* CV026. The preparations of the culture liquid of *P. aureofaciens* were used as standards; the strain synthesizes N-(3-oxyhexanoyl) homoserine and N-(3-oxobutanoyl) homoserine lactone [19]. The N-HHL synthesis by strain *P. aurantiaca* B-162 and the regulatory mutants was assayed. The synthesis of this compound was determined indirectly, from absorption changes at 585 nm of the voilacein preparation accumulated by *C. violaceum* CV026. Analysis of our results revealed that one mutant, *P. aurantiaca* B-162/57, synthesized almost twice as much N-HHL as the initial strain; this is probably the cause of its high production of phenazine antibiotics.

The cause for N-HHL overproduction by the bacteria under study is presently unclear. Increased synthesis of these molecules may possibly be related to mutations in the *rsaL* gene. A QS system negative regulator is the product of this gene; this has been demonstrated for *P. putida* WCS358 [20]. N-HHL overproduction may also result from changes in the functioning of the GacA/GacS system; it is known to be involved in the positive regulation of transcription of *phzI*; N-HHL is the product of this gene.

Thus, we propose a new method for obtaining regulatory mutants capable of overproduction of phenazine antibiotics; the method is based on the application of toxic amino acid analogues (azaserine, *m*-fluoro-DL-phenylalanine, and 6-diazo-5-oxo-L-norleucine) as selective factors. The productivity of the strains obtained according to this procedure can reach 205 mg/l, i.e., three times higher than in the initial strain *P. aurantiaca* B-162 and ten times higher than in all the other *Pseudomonas* strains studied in this respect [14, 21]. The mechanism of overproduction of phenazine antibiotics in the regulatory mutants was revealed.

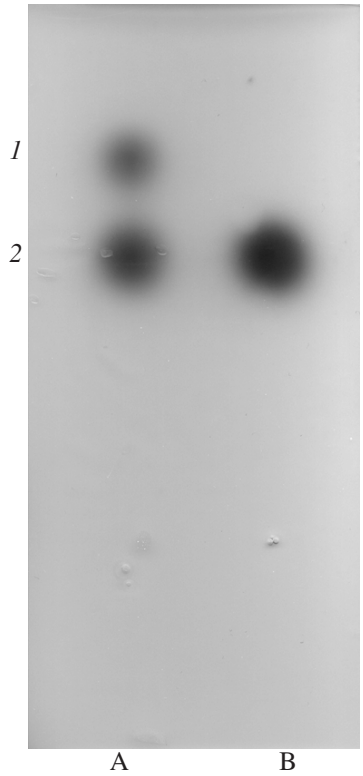

Fig. 3. Thin-layer chromatography of N-acyl-homoserine lactone samples isolated from *P. aureofaciens* B-161 (A) and *P. aurantiaca* B-162 (B), visualized by an overlay of *C. violaceum* CV026 culture: N-butanoyl-homoserine lactone (*1*); N-hexanoyl-homoserine lactone (*2*).

The phenomenon was shown to be related either to deregulation of DAHP synthase (removal of inhibition by phenylalanine, tyrosine, and phenazine) or to N-HHL overproduction (in the case of *P. aurantiaca* B-162/57).

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