Physiological engineering of *Pseudomonas aurantiaca* antimicrobial activity: effects of sodium chloride treatment

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Abstract

The influence of sodium chloride (NaCl) treatment on the antifungal activity of the bacterium *Pseudomonas aurantiaca*, a producer of biopesticide for vegetable plants, was investigated. It was shown that an increase in the NaCl concentration in incubation solutions from 1 M to 3 M led to a significant increase in the antifungal activity of this bacterium. Antifungal activity continued to increase with prolonged treatment of bacteria in fresh nutrient medium from 72 h to 96 h. These findings could be very important for the further development of biotechnological processes directed not only to the production of new active biopesticides but also of other valuable resources.

Introduction

It is well known that the global population is expected to grow very significantly in the next decades. This means that an additional increase in agricultural production will be required. However, the availability of arable area is on the decline and most soils with high productivity potential are already under cultivation. This problem can only be solved by improvements in crop productivity with appropriate control of plant pests, diseases and weeds.¹ World-wide losses of crop production due to different plant diseases are close to 13%, and such a situation significantly reduces the production of food. About 11,000 diseases are induced by 120 genera of fungi, 30 types of viruses and 8 genera of bacteria.² Currently, the control of plant diseases is achieved mainly by chemically synthesized pesticides.³ However, the use of chemical pesticides has a negative environmental impact due to the fact that these compounds may pass through the soil and subsoil and pollute surface waters and

groundwater.⁴ They are also toxic to living organisms and can negatively affect human health.⁵⁻⁷ The only way to solve this problem is to use environmentally-friendly pesticides such as biopesticides. Biopesticides are represented by live organisms or natural products derived from these organisms that suppress crop diseases. They usually offer several advantages over traditional chemical pesticides. Biopesticides are safer for the environment and they can offer much more targeted activity. They are often effective in very small quantities and decompose more quickly than conventional chemical pesticides.³ At the moment, biopesticides take up a small share of the total pesticide market; however, over the past five years, the synthetic pesticide market has been declining and, at the same time, the biopesticide market has been growing.⁸ There is a definite need for further detailed research and improvements to biopesticides to make them more efficient agricultural technologies.

Pseudomonas species are some of the most important bacteria inhabiting the rhizosphere of diverse crop plants. Soil-borne bacteria of the *Pseudomonas* genus can exert antagonistic properties on plant pathogens. They might act as efficient biological control agents able to prevent plant diseases and serve as an efficient alternative to chemical pesticides in agriculture.⁹ This is linked to their ability to produce different types of antibacterial and antifungal compounds. One of the goals of further research on microbial biopesticides is to promote the maximum activity of their active ingredients.¹⁰ This can be achieved by searching for new, more active strains or by improving the action of previously selected, existing and efficient strains. Gene engineering and metabolic engineering methods can be used to make such improvements. In spite of the great potential of these modern approaches, they also have some disadvantages. The aim of our study was to improve the activity of efficient strains of biopesticide-producing *Pseudomonas aurantiaca* by the use of a physiological approach. In the current study, we investigated the possible impact of treatment with sodium chloride (NaCl) on the synthesis of antimicrobial metabolites by *Pseudomonas aurantiaca*.

Materials and Methods

The bacterium *Pseudomonas aurantiaca* S-1 used in this study was received from the microbial collection of the Institute of Microbiology at the National Academy of Sciences of Belarus. The bacterial culture was grown in 750 mL flasks with 100 mL of Mejnell's nutrient medium which contained (in g l⁻¹): K₂HPO₄·3H₂O 7.0; KH₂PO₄ 3.0;

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MgSO₄·7H₂O 0.1; NH₄NO₃ 1.0; C₆H₅O₇·Na₃·3H₂O 0.5; molasses 30. The pH value of the nutrient medium was adjusted to pH 7.0. Bacterial cultures were cultivated using a rotary shaker at a rotation speed of 180 rpm at 30°C for 24 h. To perform the NaCl treatment, harvested bacterial biomass was then incubated in 1 M, 2 M, 3 M and 4 M NaCl solutions for 3 h at 30°C. Next, 0.5 mL of microbial culture subjected to the treatment or not subjected to the treatment (control) were taken from each solution for inoculation into fresh Mejnell's medium. After re-incubation of *Ps. aurantiaca* for 72 h or 96 h, the antifungal metabolite synthesis was measured. An evaluation of the antifungal activity of cell-free supernatants of *Ps. aurantiaca* culture medium was carried out using spectrophotometry. The absorption spectrum was measured between 300 nm and 700 nm using an Ultrospec 2100 Pro spectrophotometer with a 1 cm disposable sample cell. Pure medium was used as a reference. The size of the peak in the area of 400 nm was used for the determination of antifungal activity. All experiments were performed in five replicates, and representative results are presented. To check the validity of this method of determining antimicrobial activity, in some experiments, antifungal activ-

ity was also determined using the traditional agar well diffusion method.¹¹ In this assay, the fungus *Fusarium oxysporum* NR 259 was used as the test culture, obtained from the Microbial Strain Collection of Latvia. This culture is known to be a vegetable and fruit pathogen. The fungus *F. oxysporum* was cultivated on solid PDA (potato dextrose agar) medium for 48 h. Circular wells (6 mm) were cut in the agar culture medium and filled with 70 µL of liquid cultivation medium obtained from *Ps. aurantiaca* culture grown in Meinell's medium for 72 h or 96 h. Antifungal activity was measured as the inhibition of fungal growth from the edge of wells to the end of the fungal inhibition zone (in cm).

Results and Discussion

Our previous studies on anhydrobiosis (a temporary reversible delay of metabolism) of microorganisms achieved by significant dehydration of cells showed that cell transfer into this state is connected with a number of intracellular changes at the molecular, structural and cellular levels. It was revealed that some compounds were synthesized in the cells; this does not usually happen in their normal state. Unusually, the activation of some metabolic processes was also observed. Most interestingly, these effects were not only linked with the increased synthesis of protective compounds, but also with the synthesis of some other compounds, as well as with the activation of growth and of fermentation processes.¹²⁻¹⁶ It

was shown that the physiological state of cells can be changed or improved during incubation in solutions with increased osmotic pressure, thus creating conditions that lead to the dehydration of microorganisms.¹⁶⁻¹⁹ In our current work, we attempted to reveal if similar stress treatment can also efficiently increase the physiological antimicrobial activity of the bacterium *Ps. aurantiaca*, a producer of biopesticides. As the chemical compound used to increase the osmotic pressure, we chose NaCl because it is safe and is one of the cheapest substances available. Both these characteristics seemed, to us, to be very important in the event that NaCl treatment demonstrated positive effects and could be recommended for biotechnological application. In our experiments, we used different concentrations of this salt, from 1 M to 4 M. It is necessary to mention here that in another series of experiments that were performed simultaneously with this study, we investigated possible changes in the antimicrobial activity of *Ps. aurantiaca* as a result of another stress treatment, i.e. heat shock. In these experiments, for the determination of antimicrobial activities, we used the conventional but labour- and time-consuming agar well diffusion method and spectral analysis of cell-free supernatants of *Ps. aurantiaca* culture medium. The latter was applied on the basis of earlier results which showed that *Ps. aurantiaca* cell-free culture media supernatants present a spectral peak at 390-420 nm associated with the antifungal activity of this bacterium.²⁰ Our results from studies on the effects of heat shock showed that there was very good correlation between

these data. On the basis of these results, it was decided to use the rather convenient spectral method for the evaluation of antifungal activity of our strain of *Ps. aurantiaca* (G Khroustalyova, N Zharova, L Rozenfelde, M Mandryk, E Kolomiets, A Rapoport, manuscript in preparation). Therefore, this very convenient methodological approach was used in this study on the influence of NaCl upon the antifungal activity of *Ps. aurantiaca*. The results of this work are shown in Figures 1 and 2. Figure 1 represents the typical spectral analysis of cell-free supernatants of *Ps. aurantiaca* culture medium. To facilitate further discussion, Figure 2 shows the results of a typical experiment with changes in the height of the main absorbance peak at 390-400 nm. This was previously shown to correspond to the antifungal activity of this strain.²⁰ It can be seen from both figures that the use of inoculum previously treated with various concentrations of NaCl (1-4 M) led to an increase in the antifungal activity of the bacterial culture grown for 72 h or 96 h. It is also clear that an increase in the NaCl concentration from 1 M to 3 M definitely increased the effect of the treatment. The same effect was also revealed with an extension of the duration of bacterial growth (from 72 h to 96 h). The highest level of antifungal activity was found after 96 h of growth in the inoculum previously subjected to incubation in a 3 M solution of NaCl; this increased by up to 3.25 times compared with the activity of the control culture. To be sure of the validity of this spectral method used for the evaluation of antifungal activity, in some experiments performed simultaneously with this method, we also used

Figure 1. Typical spectra of cell-free cultural medium supernatants of *Ps. aurantiaca*, grown in fresh nutrient medium after incubation in solutions with different concentrations of sodium chloride (1 M - 4 M). The control sample was not subjected to sodium chloride treatment.

Figure 2. Absorbance at 400 nm of cell-free cultural medium supernatants of *Ps. aurantiaca*, grown in fresh nutrient medium for 72 and 96 h after sodium chloride treatments (1 M-4 M) and of the control sample which was not subjected to sodium chloride treatment.

the traditional agar well diffusion method.¹¹ Typical results of these experiments are shown in Figure 3. A comparison of the results obtained by these two methods is summarized in Table 1. It can be seen that there is a good correlation between the results obtained by each of these two methods. Of course, the figures also show that there are some differences and it seems that the use of the spectral method sometimes provides slightly more positive results than the traditional agar well diffusion method. At the same time, the tendency for an increase in antifungal activity after the treatment of bacteria with NaCl solutions as well as the dependence of such an increase on the concentration of the solution can clearly be seen with the use of any of the applied meth-

ods. So, in summary, our results reveal very significant activation of the metabolism of a bacterial culture of *Ps. aurantiaca* that can then be used as an inoculum for the production of antifungal compounds. This activation of metabolism was linked with an increase in the concentration of NaCl used for the incubation of bacteria and, correspondingly, with an increase in the osmotic pressure of the solution. It is clear that these conditions induce preliminary dehydration in microbial cells that, of course, does not lead to a delay in metabolism (anhydrobiosis) but rather a change in intracellular metabolism in the direction of activation of a number of intracellular protective reactions for the maintenance of cell viability.

Conclusions

We propose that it would be very interesting to study the reasons why, under these conditions, protective reactions take place as well as the activation of metabolic pathways that are not directly connected to the maintenance of cell viability. As previously mentioned, we have also shown the activation of other metabolic routes which are also not directly connected with cell viability.¹³⁻¹⁴ Nevertheless, the present results show that such stress treatment to cells of *Ps. aurantiaca* leads to the activation of *cryptic* metabolic pathways. In 1994, Jens Nielsen proposed a new term: *physiological engineering*.²¹ We propose that the effects of the stress treatments shown in this study can also be considered as an approach to physiological engineering, and that this method could be very efficient not only for the production of biopesticides by *Ps. aurantiaca*, but in various other biotechnological processes as well.

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Figure 3. Antifungal activity of cell-free cultural medium supernatants of *Ps. aurantiaca*, grown in fresh nutrient medium for 96 h after sodium chloride (NaCl) treatments (1 M - 4 M) and of the control sample against the phytopathogenic fungus *F. oxysporum*. 1 - 1 M, 2 - 2 M, 3 - 3 M, 4 - 4 M, C - reference sample (control). Results were obtained with the use of the agar well diffusion method.

Table 1. Comparison of the results on the antifungal activity of cell-free cultural medium supernatant of *Ps. aurantiaca*, grown for 96 h, obtained by the spectral method and agar well diffusion method.

Sample	Spectral method		Agar well diffusion method	
	Absorbance at 400 nm	Absorbance increase to control, times	Fungus growth inhibition zone size, cm	Inhibition zone size increase to control, times
Control	0.4	-	0.30	-
1 M NaCl	0.5	1.25	0.43	1.43
2 M NaCl	1.1	2.75	0.65	2.16
3 M NaCl	1.3	3.25	0.82	2.73
4 M NaCl	0.8	2	0.61	2

NaCl, sodium chloride.

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