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Different Types of Nitrogen Stress Response in Plant Pathogenic Bacterium *Pectobacterium Atrosepticum*

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Research Article

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ABSTRACT

Bacterial stress response under adverse conditions was long considered to be associated with the decrease of the population density because of the death of significant portion of the cells. However, recently in our studies by the example of carbon starvation of plant pathogenic *Pectobacterium atrosepticum* SCRI1043 it has been demonstrated that in the cases when the bacterial population density is low (below quorum level) the adaptation strategy may be related to cell proliferation and increase of cell number. Such kind of strategy enables low amount of cells to form functional population and implement intercellular communication necessary to resist stress factors. In the present study we show that the optimization of the population density and cell proliferation under stress conditions occurs not only during carbon starvation, but also in the course of nitrogen deficiency. Depending on initial population density, nitrogen stress response is realized according to alternative scenarios (related to either increase or decrease of cell titre) that have both common and distinctive features between themselves and the resembling reactions induced by carbon starvation.

INTRODUCTION

Bacteria have high adaptive potential that provides their survival under various adverse conditions. There are two general types of adaptation strategies chosen by bacteria depending on the density of their population. The first conversional strategy related to the arrest of cell proliferation, lysis of a part of the population and formation of various types of adaptive forms is realized when the population density at the beginning of stress effect is high [1,2]. The second one takes place if the population density is low (below the quorum level). Herewith, the increase of the population size occurs until the threshold value of ~106 cells per mL, when the quorum sensing and stringent response are activated. The latter strategy was demonstrated by the example of carbon starvation of *Pectobacterium atrosepticum* (*Pba*)–a gram-negative phytopathogenic bacterium [3,4], *E. coli* [5] and many other species of gram-negative and gram-positive bacteria (our unpublished data) and thus may be considered to be universal among different bacteria.

In the present study, we examined if the phenomenon of cell division under stress condition is a specific reaction realized during the deficiency of exogenous carbon or a strategy that provides the ability to resist the lack of other nutrients (nitrogen). We also compared *Pba* adaptation strategies induced by nitrogen and carbon starvation in terms of the formation of cross-protection and expression of stress-related genes. Additionally, the transcript levels of genes related to nitrogen assimilation were compared in starving cultures of high and low initial population densities.

MATERIALS AND METHODS

Bacterial Strains, Media and Culture Conditions

A strain of *Pectobacterium atrosepticum* SCRI1043 (formerly *E. carotovora ssp. atroseptica* SCRI1043) (*Pba*) [6] was grown in either LB (Luria-Bertani) medium or D5 medium (100 mM sodium phosphate buffer, pH 7.5, 1 g L^{-1} NH_4Cl, 0.3 g L^{-1} $MgSO_4$ ×

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$7H_2O$ and 2 g L^{-1} sucrose) with aeration (200 r.p.m.) at 28°C. To obtain nitrogen starving cultures, *Pba* cells grown in LB medium were harvested (8,000 g, 10°C, 10 min) at the early stationary growth phase, then washed twice in NH4Cl-depleted D5 medium, resuspended in NH4Cl-depleted D5 medium with a range of initial population densities of 10^2-10^9 CFU per ml and incubated in glass vials without aeration at 28°C. The titre of colony-forming units (CFUs) was determined by plating serial 10-fold dilutions of the cell suspensions in 0.5% NaCl onto 1.5% LB agar after 0, 4, 8, 12, 18, 24, 48 and 72 hours of incubation. Media used in this study were autoclaved at 121°C for 40 min..

Sucrose uptake assay

To trace the consumption of exogenous carbon substrate (sucrose) in growing and nitrogen-starving *Pba* cultures, cell-free supernatants were collected. To obtain supernatants, a bulk of the cells was removed from the cultures by centrifugation (14,000 g, 10°C, 10 min); the remaining cells were removed by filtration through nitrocellulose filters (0.2 µm; Millipore, Germany). Carbohydrate content in the cell-free supernatants was measured using the phenol-sulphuric acid assay [7].

Cross-protection assay

To assess the cross-protective effect of nitrogen starvation stress (primary stress), the tolerance of starving (or non-starving) *Pba* cells toward heat shock, hydrogen peroxide and NaCl (secondary stress) was examined. Prior to secondary stress, the cell density of different *Pba* cultures was adjusted to ~10^6 CFU ml^{-1} by diluting in nitrogen-free medium. For secondary stress challenge, 1 ml aliquots of cell suspensions were subjected to 50°C for 5 min, or supplemented with 20% NaCl or 2.5 mM H_2O_2. Before and after 5 min, 4, 8 and 24 h of exposure to one of the secondary stress factors (heat shock, or H_2O_2, or NaCl), suspensions were plated onto 1.5% LB agar as serial 10-fold dilutions. Plates were incubated at 28°C for two days and the CFUs were counted. Bacterial densities were expressed as a log CFU per ml.

Virulence assay

Virulence of early stationary phase and nitrogen starving *Pba* cells was determined by their ability to cause symptoms of disease on potato plants (*Solanum tuberosum* cv. Desiree). Plants were vegetatively propagated and grown axenically in test tubes containing MS medium with 0.3% agar [8] with a 16 h light/8 h dark cycle photoperiod for 15-20 days. Thereafter, plants were inoculated with *Pba* cells of cultures starving during four days at high or low initial population densities or early stationary phase *Pba* cells. The titer of the inoculum was adjusted to 10^7 CFU ml^{-1} for all tested cultures by concentrating using centrifugation or diluting in nitrogen-free medium. Ten microliters of the obtained suspensions (105 CFU per inoculum) were placed on slightly wounded stem using a sterile syringe. The percentage of the plants, which displayed pronounced disease symptoms or died, was determined 7 days post-inoculation. The presented data are the values obtained in two independent experiments performed in 20 biological replicates each.

Gene Expression Analysis

Bacterial RNA was extracted after 0, 4, 8, 24 and 48 hours of incubation of *Pba* cells under nitrogen-free conditions using RNAExtract reagent (Eurogen, Russia) according to manufacturer's instructions. Total RNA was treated with Turbo-DNAse, quantified using a Qubit fluorometer (Invitrogen, USA) and used for cDNA synthesis. The reaction mixture for reverse transcription contained 100 pmol random hexamers, 1 mM dNTPs, 200 U RevertAid reverse transcriptase (Thermo Scientific, USA) with the corresponding 1 × reaction buffer. First, water and random hexamers were mixed with RNA and incubated for 5 min at 70°C and cooled immediately on ice. The other components were then added. Incubation was performed using a DNA Engine thermocycler (Bio-Rad, Hercules, CA, USA). Reverse transcription was performed as follows: 10 min at 25°C, followed by 1 h at 42°C and 10 min at 70°C. Two µl of cDNA were used as the template for qPCR.

Real-time RT-PCR was performed using EVA Green Reaction mixture (Synthol, Russia). Primers **(Supplementary Table 1)** were designed according to the genome sequence of *P. atrosepticum* SCRI1043 (NCBI Gen-Bank accession number NC_004547) using Vector-NTI Version 9 software (Invitrogen, Carlsbad, CA, USA) and synthesized by Synthol (Moscow, Russia). PCR was performed under the following conditions: 95°C for 2 min, followed by 45 cycles at 94°C for 10 s, 60°C for 15 s and 72°C for 30 s. After that, the melt curve analysis was performed in a temperature range of 60-95°C. The reactions were run and changes in fluorescence emission were detected using a CFX96 quantitative PCR system (Bio-Rad, USA). The amount of fluorescence was plotted as a function of the PCR cycle using CFX Manager Software (Bio-Rad, USA). The amplification efficiency (E) for all primers was determined using a dilution series of a pool of cDNAs. Additional controls included the omission of reverse transcriptase to measure the extent of residual genomic DNA contamination and omission of template. The expression level of target genes was calculated relative to reference genes. The geNorm software was used to choose genes that displayed stability in the expression under the experimental conditions. Among six (*gyrB*, *groES*, *tufAB*, *ffh*, *rpoD* and *recA*) candidate reference genes *tufAB*, *recA* and *ffh* had the most stable expression levels; therefore these genes were used as the reference ones. Three replicates were performed for each reaction. Relative expression levels were determined as the ratios between the quantities of cDNA corresponding to the target genes and values of normalization factor, which was calculated using geNorm software. The presented data were obtained by the analysis of three independent experimentss.

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Statistical Analysis

The experiments were performed in three biological replicates, each one assayed in technical triplicate. The significance of differences in results of each test and relative control values was determined using Student's t-test.

RESULTS

The Dynamics of CFU titre and Organic Carbon Consumption under Nitrogen Deficiency

In the nitrogen-starving *Pba* cultures of high initial cell density, the number of cultureable cells gradually decreased **(Figure 1)**. Herewith, the content of extracellular carbohydrates decreased only within first hours of starvation and further remained at the level of around 40% of initial amount indicating that the consumption of sucrose occurred only transiently during the starvation **(Figure 2)**. In turn, when the *Pba* cells were transferred to nitrogen-deficient medium with low population densities, the number of cells increased until reaching a value of around 10^6 cells per mL **(Figure 1)**. The alteration in the content of extracellular carbohydrates in this case was not observed **(Figure 2)**.

When the nitrogen-deficient medium was supplemented with 1 g/L of NH_4Cl, the *Pba* growth proceeded until the population density reached the values of 1-2 × 10^9 CFU ml^{-1} irrespective of whether the initial cell density was high (1.1 × 10^8 ± 9.8 × 10^6 CFU ml^{-1}) or low (1.4 × 10^4 ± 1.1 × 10^3 CFU). Herewith, in the course of growth, the level of extracellular organic carbon considerably decreased and only trace amounts of extracellular carbohydrates were detected after three days of cultivation **(Figure 2)** pointing to a full utilization of exogenous substrate.

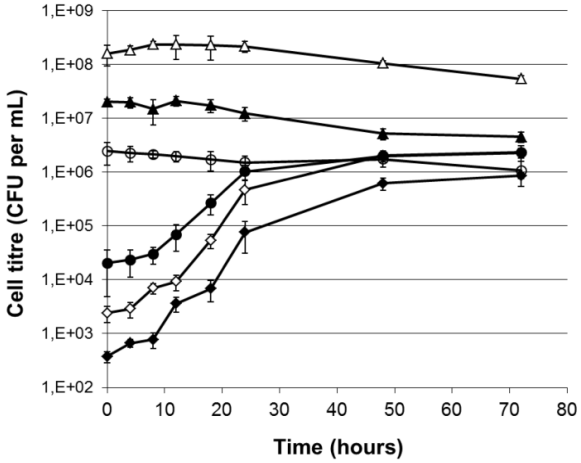

Figure 1. The dynamics of CFU titre in nitrogen-starving P. atrosepticum cultures of different initial population densities: 1.6 × 10^8 CFU mL^{-1} (white triangles), 2.0 × 10^7 CFU mL^{-1} (black triangles), 2.5 × 10^6 CFU mL^{-1} (white circles), 2.0×10^4 CFU mL^{-1} (black circles), 2.4×10^3 CFU mL^{-1} (white diamonds), 3.8×10^4 CFU mL^{-1} (black diamonds). The presented data are average ± SD of three independent experiments.

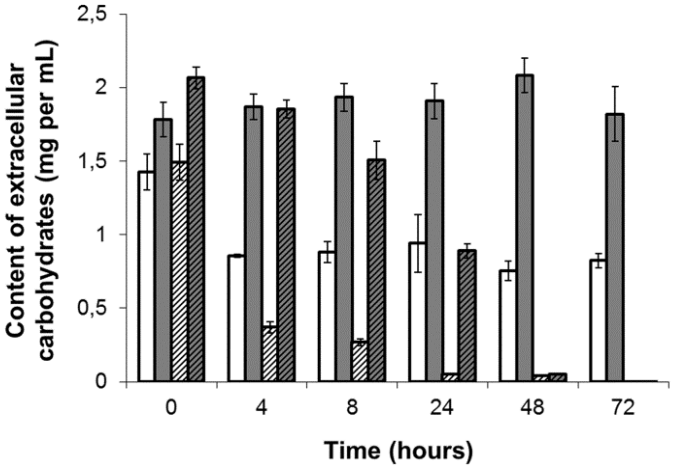

Figure 2. The content of exogenous growth substrate (carbohydrates, sucrose) in the supernatants of P. atrosepticum cultures incubated in the absence of exogenous nitrogen (non-hashed columns) and grown in minimal D5 medium (hashed columns). The media were inoculated with high (1.1 × 10^8 ± 9.8 × 10^6 CFU ml^{-1}, white columns) or low (1.4 × 10^4 ± 1.1 × 10^3 CFU ml^{-1}, grey columns) population densities. The presented data are average ± SD of three independent experiments.

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Resistance and Virulence of Nitrogen-starving *Pba* Cells

To ascertain if nitrogen starvation induces the formation of cross-protection to secondary stress factors, the growing and starving *Pba* cells were subjected to hydrogen peroxide, osmotic and heat shocks. In the suspensions of early stationary phase cells, the addition of H_2O_2 (2.5 mM) led to continuous reduction of CFU titre up to undetectable level after 24 h of incubation **(Figure 3A)**. Although H_2O_2 treatment of nitrogen starving cells was also coupled with the reduction of cell titre right after the exposure, the number of CFUs gradually restored throughout the incubation **(Figure 3A)**. The resistance to H_2O_2 was acquired by the cells of starving cultures inoculated at both high and low initial population densities.

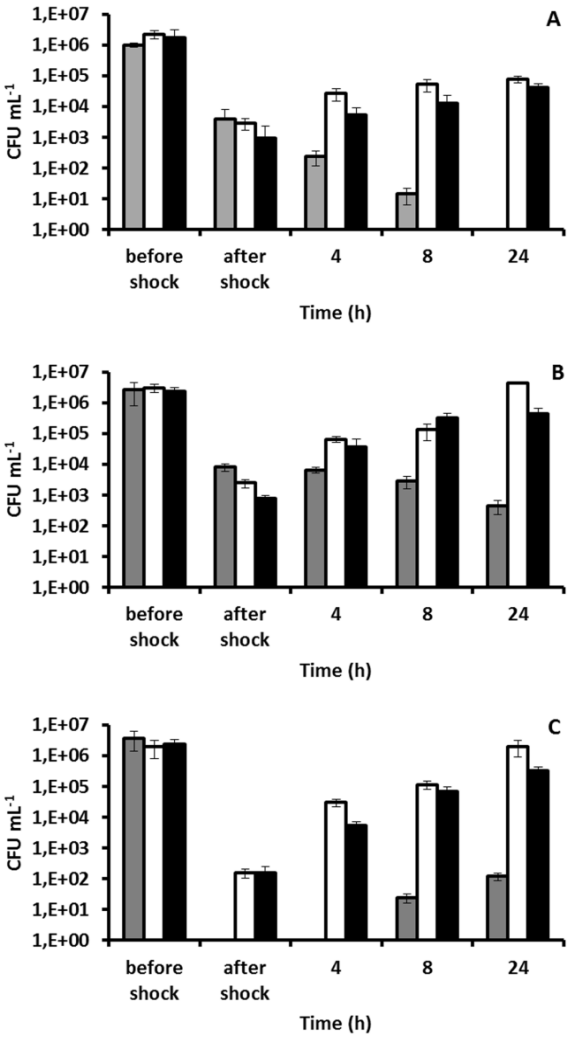

Figure 3. The resistance of early stationary phase and 4-day nitrogen-starving P. atrosepticum SCRI1043 cells to: (A) 2.5 mM of hydrogen peroxide; (B) 20% NaCl; (C) heat shock (50°C). Grey columns–early stationary phase cells, white and black columns–starving cells incubated at high and low initial population densities, respectively, in nitrogen-deficient medium during four days. Prior to the start of the experiment, the CFU titre of starving and early stationary phase cell cultures was adjusted to ~106 CFU ml^{-1} by diluting in nitrogen free medium. Values represent means ± SD of three independent experiments.

After heat shock at 50°C for 5 min, the CFU titer of early stationary phase cell suspensions became null during first hours after the exposure; 8 and 24 h after the shock the CFU titers constituted only 0.02 and 0.1% of the initial titre, respectively **(Figure 3B)**. In the suspensions of nitrogen starving cells, after heat shock, the CFU titre did not decrease to undetectable level and restored much faster than that in the suspensions of growing cells **(Figure 3B)**. Incubation of growing cells in the presence of 20% NaCl was associated with a gradual decrease of CFU titer to the level of 0.2% of the initial one. In turn, although NaCl treatment of nitrogen starving *Pba* cells led to the reduction of CFU titer right after the exposure, the cell number gradually restored throughout the incubation to almost initial level **(Figure 3C)**. The increased heat shock and NaCl resistance was a characteristic of nitrogen starving *Pba* cells irrespective of whether the initial cell density of the starving cultures was high ($1.1 \times 10^8 \pm 9.8 \times 10^6$ CFU ml^{-1}) or low ($1.4 \times 10^4 \pm 1.1 \times 10^3$ CFU).

To check if nitrogen-starving *Pba* retain virulence, potato plants were infected by growing log phase cells as well as four

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day nitrogen starving cells of the cultures of either low or high initial population densities. Sixty two percent of plants infected by growing *Pba* cells died or displayed severe disease symptoms 7 days after the inoculation. Herewith, 100% and 88% of plants were significantly damaged by nitrogen starving *Pba* cells incubated at low and high initial cell densities. This indicates that nitrogen starving *Pba* cells not only retain their virulence but become even more aggressive.

The Expression of Stress Response- and Nitrogen Assimilation-related Genes under Nitrogen Deficiency

The expression levels of *relA* (encoding synthase of ppGpp), *spoT* (encoding bifunctional synthate/hydrolase of ppGpp), and rpoS (encoding alternative stress-induced sigma-factor) genes were examined in the course of nitrogen starvation. The induction of the expression of these genes was widely shown to be associated with stress response under carbon deficiency and other stress effects [9,10]. However, during nitrogen starvation of *Pba* cells, the induction of the expression of neither *relA* nor *spoT* nor *rpoS* was observed (data not shown).

The expression levels of *nifD* and *nifK* genes encoding α- and β-subunits of nitrogenase as well as *nifH* gene encoding a reductase, which transfers electrons from a suitable donor, such as reduced ferredoxin (flavodoxin), to nitrogenase catalytic centre during nitrogen assimilation were induced in the course of nitrogen starvation in both high and low cell density starving cultures **(Figure 4)**. The assimilation of nitrogen is known to be controlled via global transcriptional regulator NtrC (GlnG) or regulatory protein *NifA* or both [11,12]. The expression level of *glnG* gene in *Pba* was decreased during nitrogen starvation irrespective whether the inoculation titre was high or low **(Figure 4)**. In turn, the increased transcript level of *nifA* gene was observed in starving cultures of high population density; herewith, in those of low cell density, the expression level of this gene was decreased **(Figure 4)**.

The assimilated nitrogen in bacteria may be incorporated into either amino acids via the action of glutamine synthetase *GlnA* and glutamate dehydrogenase GdhA or pyrimidines by carbamoyl phosphate synthase, two subunits of which are encoded by *carA* and *carB* genes. The expression levels of *glnA* and *gdhA* genes were decreased in nitrogen starving *Pba* cultures **(Figure 4)**. Herewith, the transcript levels of *carA* and *carB* were increased in the course of nitrogen starvation in the cultures inoculated at both high and low titre **(Figure 4)**. This indicates that carbamoyl phosphate synthase-dependent pathway of ammonium incorporation into nucleotides was induced in nitrogen starving *Pba* populations.

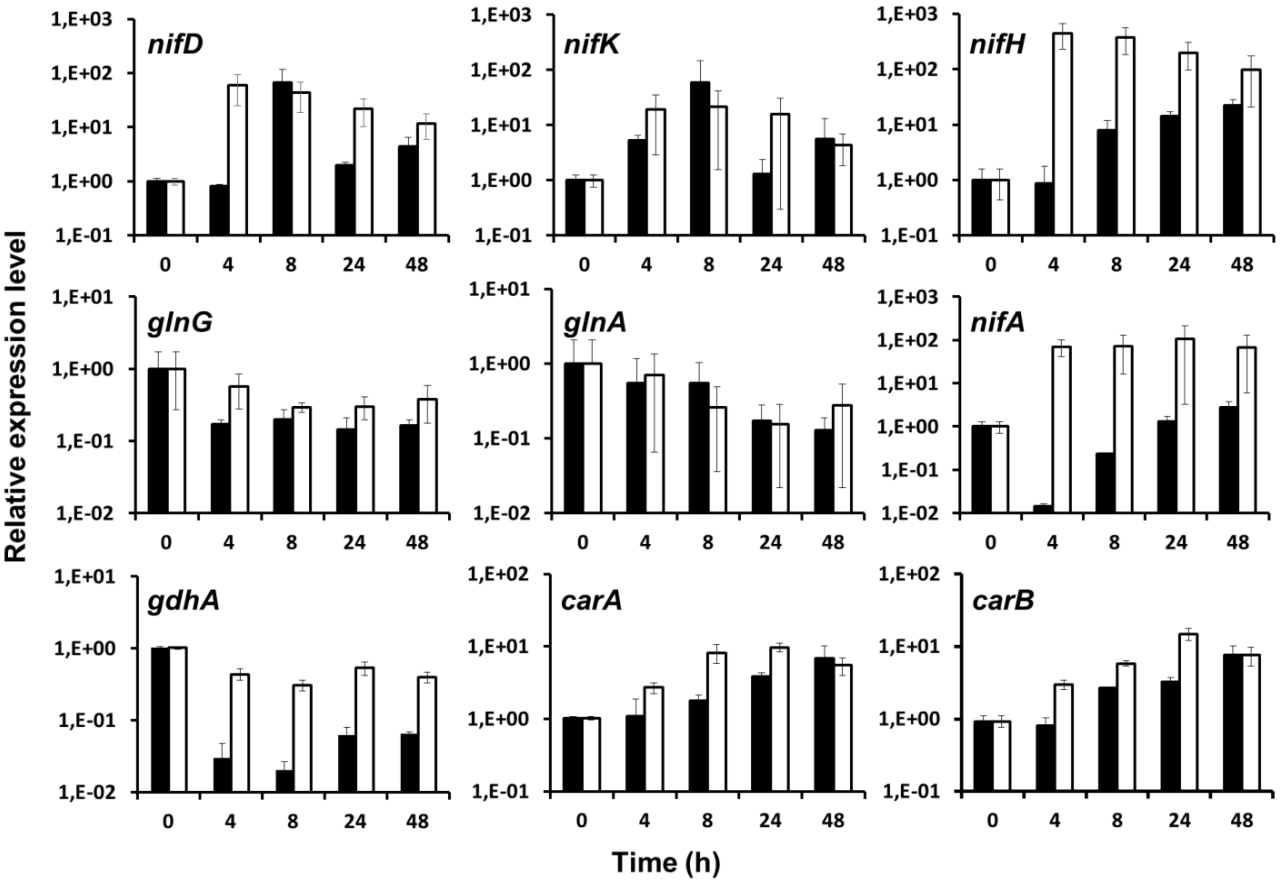

Figure 4. The dynamics of the expression level of genes, which products are involved in nitrogen assimilation, in the nitrogen-starving cultures of *P. atrosepticum*. Cells were subjected to nitrogen starvation at low (black columns) or high (white columns) population densities. The values represent means ± SD for three biological replicates each analyzed in technical triplicates.

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DISCUSSION

In the present study we have shown that the increase in bacterial population density under stress conditions is not just a specific response to carbon starvation; such kind of a strategy is also realized when the nitrogen substrate is depleted from the bacterial microenvironment. When bacteria are subjected to nitrogen starvation at high population density, they realize conversional strategy of stress response associated with the decrease in cell titre that may be related to the lysis of a part of the population or formation of dormant cells or both. Herewith, a significant portion of exogenous carbon substrate (sucrose) remains in the cultivation medium and is not consumed by nitrogen starving bacteria. In turn, if the population density is low under nitrogen deficiency, an increase of the cell titre occurs up to a level of 106 CFU per mL same as during carbon deficiency that was reported in our previous studies [3,4].

Carbon deficiency is known to increase bacterial resistance to various secondary stress factors providing the formation of so called cross protection [13]. The effect of cross protection in *Pba* cells in the course of carbon starvation is achieved irrespective of whether the cells are subjected to stress effect under high or low population densities [4,14]. However, under nitrogen deficient conditions, the formation of cross protection in bacteria has not been previously demonstrated. In our experiments we have shown that the alternative reactions (related to either decrease or increase of population densities) induced by nitrogen deficiency do enhance cell resistance to various stress factors conferring cross protection. Moreover, under nitrogen starvation, *Pba* cells retain virulence and even more become more aggressive than actively proliferating cells.

The formation of cross protection under carbon deficiency is known to be associated with the increased expression of specific genes that encode proteins necessary for the realization of stress response – synthase RelA and synthase/hydrolase SpoT of guanosine tetraphosphate (ppGpp) and alternative stress induced sigma-38 factor RpoS [13,15]. In *Pba*, the formation of cross protection under carbon deficiency is also associated with the increased transcript levels of these genes [4]. However, under nitrogen deficiency the expression levels of relA, spoT and rpoS genes were not increased although the cells did acquire cross protected phenotype. Despite that ppGpp and RpoS in many cases have been shown to be required for the stress response and the formation of cross protection, there are several evidences for the existence of ppGpp- and RpoS-independent mechanisms of bacterial adaptation [10,16-20]. To date, ppGpp and RpoS-independent mechanisms of bacterial adaptation remain enigma. Thus, our data indicate that the formation of cross protection in carbon [4] and nitrogen (this study) starving *Pba* cells are presumably achieved via different regulatory pathways.

One of the distinctions of bacterial stress response under carbon and nitrogen deficiency consists in activation of so called nitrogen stress response – an adaptive mechanism used by bacteria to scavenge for alternative nitrogen sources [12]. Nitrogen stress response is coupled with atmospheric nitrogen assimilation that is carried out by nitrogenase. Besides diazotrophic microorganisms, nitrogenase complex is present in many representatives of *Enterobacteriaceae* family: *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Erwinia herbicola*, *Citrobacter intermedius* [21-23]. In *Pba*, molybdenum-iron nitrogenase typical of most diazotrophic microorganisms was revealed [24].

In our experiments, genes encoding two subunits of *Pba* nitrogenase as well as NifH reductase were up-regulated under nitrogen starvation irrespective of whether the inoculation titre was high or low. The expression of these genes is controlled by global transcriptional regulator NtrC (GlnG), which induces master regulator of nitrogen fixation NifA that activates nitrogen assimilation [25]. Induction of nifA gene was also shown to occur independently of NtrC (GlnG) [11,26,27]. In our experiments, no activation of glnG expression occurred in both analyzed types of nitrogen starving *Pba* cultures. Nevertheless, in high cell density cultures, *nifA* gene was highly induced in the course of nitrogen starvation. Herewith, no increase in the transcript level of this gene has been observed for the starving cultures inoculated at low cell density. These results point to NtrC-independent activation of nitrogen stress response in *Pba* under the described above conditions. In high cell density cultures this response is likely mediated *via NifA*; the regulators engaged in the coordination of nitrogen stress response under low population density remain unknown. Thus, depending on the type of nitrogen stress response (related to either increase or decrease of the cell titre depending on the initial population density) *Pba* are likely to utilize alternative regulatory mechanisms in order to adapt to stressful conditions.

Nitrogen assimilated by bacteria is known to be included into biomass within either amino acids or nucleotides [28]. The genes encoding the enzymes responsible for the incorporation of nitrogen into amino acids were not induced under nitrogen starvation of *Pba*. Herewith, *carA* and *carB* genes encoding carbamoyl phosphate synthase were up-regulated in nitrogen starving cultures irrespective of the inoculation titre. This means that the assimilated nitrogen in *Pba* under the assayed conditions is incorporated into nucleic metabolism.

Taken together, our investigations show that the nitrogen stress response may be represented by two distinct types. The differences in two types are related to dynamics of the population density (increase or decrease) depending on the inoculation titre, and different regulators involved in the activation of the nitrogenase complex. Common features of two adaptive strategies are related to the formation of the cross protected phenotypes, induction of nitrogenase complex in NtrC-independent manner and up-regulation of genes, which products are involved in incorporation of assimilated nitrogen into nucleotides. The described

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adaptive nitrogen-deficiency-induced optimization of the cell density is not associated with the activation of stringent response-related genes and *rpoS* gene unlike the resembling reactions occurring under carbon starvation..

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