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The Role of Mineral Phosphorus Compounds in Naphthalene Biodegradation by *Pseudomonas Putida*

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Abstract—The effect of phosphate concentration in the culture medium on the growth and naphthalene degradation by *Pseudomonas putida* BS 3701 was studied. The limiting concentration of phosphate was 0.4 mM and 0.1 mM under cultivation in media with naphthalene and glucose, respectively. The phosphate deficiency correlated with a decrease in the activities of naphthalene dioxygenase and salicylate hydroxylase and with salicylate accumulation in the culture medium. We suggest that this fact indicates the impaired regulation of gene expression of "upper" and "lower" pathways of naphthalene oxidation. Under naphthalene degradation, the cells accumulated three times more inorganic polyphosphates as compared with the consumption of glucose. The involvement of polyphosphates in the regulation of naphthalene metabolism has been considered.

Keywords: *Pseudomonas putida*, phosphate, polyphosphates, naphthalene, biodegradation

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Inorganic polyphosphates, linear polymers of orthophosphoric acid, are not only reserve phosphorus compounds of microorganisms but are also important regulators involved in the switching of genetic programs and stress adaptation in bacteria [1–3]. Biodegradation of exogenous compounds requires significant restructuring of the bacterium metabolism, with a large group of genes being expressed. It has been shown that, in degrading polychlorinated biphenyl, the *Pseudomonas* sp. strain B4 accumulates large electron–dense granules mainly consisting of phosphates, according to the data of X-ray microanalysis [4]. An increase in the content of polyphosphates was proven by an enzymatic method. It is believed that a parallel increase in the content of the GroEl stress protein and reactive oxygen species is evidence of the involvement of polyphosphates in the adaptation of bacteria to the consumption of polychlorinated biphenyl, which serve not only as a carbon source but also as chemical stress factors [4].

P. putida KT2440 mutants deficient in polyphosphate kinase, the main enzyme synthesizing polyphosphates in bacteria, differed not only by the decreased content of polyphosphates (down to 15% of the control); they were also more sensitive to ultraviolet, p-lactam antibiotics, and Cd^{2+}, and Cu^{2+} [5]. *P. putida* KT2440 contained the pWWO plasmid for toluene biodegradation, which provides for the degradation of m-xylene. In cultivation with this substrate, the strain mutant in the polyphosphate kinase gene had a longer lag-phase as compared to the parent strain [5].

These data suggest that the interrelation between the polyphosphate metabolism and biodegradation of foreign compounds by bacteria provides for cell adaptation to the assimilation of hard-to-reach and toxic sources of carbon. Thus, it is reasonable to reveal the features of phosphate consumption by bacteria from the media and those of the accumulation of inorganic polyphosphates during the biodegradation of such compounds.

The purpose of the study is to investigate the effect of phosphate in the culture medium on the growth of *P. putida* BS 3701 cells and the characteristics of polyphosphate accumulation during naphthalene degradation.

METHODS

The *P. putida* BS 3701 strain, which is capable of degradation of polycyclic aromatic hydrocarbons, was used in the study. The strain was obtained from the collection of the Laboratory of Plasmid Biology of the Skryabin Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (SIBPM, RAS).

To cultivate the bacteria, Evans (E) mineral medium was used with a pH of 7.0–7.2 and the following composition (mM): K_2HPO_4, 50; NH_4C1, 5; Na_2SO_4, 0.1; $MgCl_2$, 0.062 (μM); $CaC1_2$, 1.0; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.005 [6]. The medium was

introduced with 1.0 mL/L of a solution of trace elements in 1% HCl with the following composition (g/L): ZnO, 0.41; $FeCl_2 \cdot 6H_2O$, 5.4; $MnCl_2 \cdot 4H_2O$, 2.00; $CuCl_2 \cdot 2H_2O$, 0.17 $CoCl_2 \cdot 6H_2O$, 0.48; and H_3BO_3, 0.06. The Evans medium with 0.4 mM and 0.1 mM K_2HPO_4 contained 50 mM tris–HCl buffer with a pH of 7.2. To obtain inoculum, agar E medium with 50 mM potassium phosphate and 20 g/L of agar (Pronadisa, Spain) was used. The energy and carbon sources were glucose and naphthalene (1.0 g/L). In the growing of the bacteria on the agar medium, naphthalene was introduced onto the cover of a Petri dish.

The culture growth was assessed by optical density at 540 nm. Moreover, the number of cells in the culture was determined by plating corresponding dilutions on LB agar [7].

Salicylic acid concentration was determined by a Sintra 6 UV spectrophotometer (Australia) as was described in [8]. Supernatant samples of the cultures studied (0.5 mL) were diluted by the addition of distilled water up to 3 mL, 0.5 mL of 5% $Fe(NO_3)_2$, and 0.5 mL of 1% HNO_3; the samples were then incubated at room temperature for 10 min, and the optical density of the solution was measured at 540 nm.

Enzyme activity was determined in cell-free extract. Cells of the culture grown on the medium with naphthalene were precipitated using a K-26D centrifuge (Janetzki, Germany) at 5600 g for 10 min at 0°C, washed twice with 0.05 M potassium phosphate buffer, pH 7.0, and resuspended in the same buffer. The cell suspension was frozen and disrupted by an SIBPM press (Russia). After the disintegration, the cell debris and undestroyed cells were removed by centrifugation (39 000 g, 0°C, 60 min) using a Beckman J2-21 centrifuge (Beckman Instruments, United States). The supernatant was immediately used to determine the enzyme activity. For this, 100 μL of the extract was introduced into the reaction mixture (a final volume of 3 mL). The determination was performed at 30°C using a UV–160A spectrophotometer (Shimadzu, Japan).

The activity of naphthalene dioxygenase (EC 1.14.12.12) was determined by NADH, including measurement of a decrease in the optical density at 340 nm of the reaction mixture, which contained 0.05 M phosphate buffer, pH 7.5, 100 μM NADH, and 100 μM naphthalene that was introduced in the form of alcoholic solution [9].

The activity of salicylate hydroxylase (EC 1.14.13.1) was determined by a decrease in the optical density at 340 nm in a reaction mixture with the following composition: 0.05 M of phosphate buffer, pH 7.0, 100 μM NADH, and 100 μM of sodium salicylate [10].

The activity of catechol 2,3-dioxygenase (EC 1.14.13.2) was determined by the rate of α-oxymuconic semialdehyde formation in a reaction mixture (at 375 nm, $\varepsilon = 33.4\ mM^{-1}\ cm^{-1}$) containing 0.5 mM catechol and 0.05 M Tris–HCl buffer (pH 7.5) [11].

The activity of catechol 1,2-dioxygenase was determined by the rate of *cis,cis*-muconate formation in a reaction mixture (at 260 nm, $\varepsilon = 16.9\ mM^{-1}\ cm^{-1}$) containing 5 mM Na-EDTA, 1 mM catechol, cell–free extract, and 0.05 M phosphate buffer (pH 7.0) [12].

The specific activity of the enzymes was expressed as micromoles of the consumed substrate or as the product formed in 1 min per 1 mg of the protein.

The protein was determined using the Bradford assay [13].

The content of acid-soluble and acid-insoluble polyphosphates in *P. putida* BS 3701 cells was determined at the stationary phase of growth [2]. The biomass was separated by centrifugation at 5000 g for 1 h and washed using 0.85% NaCl. An acid-soluble fraction was obtained through treatment of the biomass with 0.5 N $HClO_4$ at constant stirring for 15 min at 0°C (per 1 g of the biomass 1.0 mL of 1.0 N $HClo_4$ and 9 mL of 0.5 N $HClO_4$). The obtained suspension was centrifuged at 5000 g for 10 min. The extraction was repeated twice, and the supernatants were united. The amount of polyphosphates was determined by the content of labile phosphorus, which was calculated using the difference in the orthophosphate content in the extract before and after hydrolysis in 1 N HCl for 10 min at 100μC [2].

The sediment of the biomass remained after the extraction was supplemented by 5–10 mL of 0.5 M $HClO_4$, heated on a water bath for 20 min at 90°C with periodic stirring an then, centrifuged in the mode mentioned above. The extraction was repeated twice, and the supernatants were united. The amount of acid-insoluble polyphosphates was determined by the content of orthophosphate found in the supernatant. The orthophosphate was determined according to [2].

Polyphosphates were determined in vivo by staining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, United States) having a maximum of the fluorescence at 456 nm [14]. A DAPI solution of 5 μL (1.0 mg/mL) was added to 100 μL of cell suspension. The cells were incubated with fluorescent dye for 30 min at 2°C, and the fluorescence was then analyzed using an Axio ImagerA1 fluorescence microscope (Zeiss, Germany) equipped with an AxioCam MRc Zeiss video camera (Germany) connected to a computer. In the presence of polyphosphates, the fluorescence maximum was shifted up to 525 nm. The DAPI–DNA complex had blue fluorescence, while DAPI–polyphosphates had yellow or orange fluorescence.

RESULTS AND DISCUSSION

It is known that phosphate fertilizers are used to accelerate biodegradation of hydrophobic compounds [15]; however, the mechanism of their stimulating effect is still unclear. Evans medium contains 50 mM

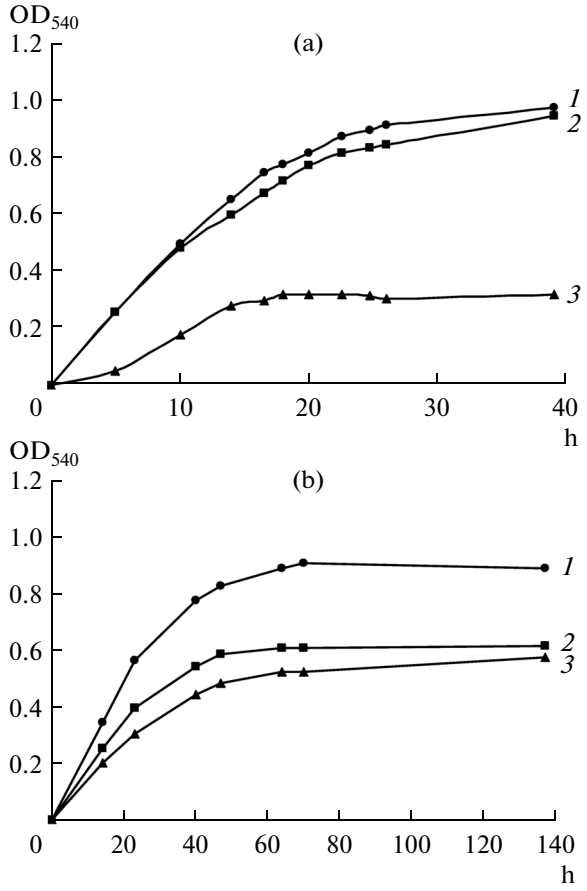

Fig. 1. Effect of phosphate concentration on growth of the *P. putida* BS 3701 strain under batch cultivation in liquid Evans medium with glucose (a) and naphthalene (b): (*1*) 50 mM phosphate, (*2*) 0.4 mM phosphate, and (*3*) 0.1 mM phosphate.

phosphate, which is necessary for the maintenance of a neutral pH, since metabolism products accumulate in the medium during cultivation of the bacteria of the *Pseudomonas* genus, which leads to acidulation [16]. Batch cultivation in mineral Evans medium with glucose or naphthalene containing different concentrations of phosphate (50, 0.4, 0.1 mM) was performed to determine the effect of phosphate on the growth of *P. putida* BS 3701. In media with a low phosphate content, 50 mM tris–HCl buffer with a pH of 7.2, was used to maintain a neutral pH.

As can be seen from Fig. 1, the phosphate demands for *P. putida* growth depended on the cultivation medium. Cultivation in medium with glucose and 0.04 mM phosphate was sufficient to reach optical density of the culture that was close to maximal. After 39 h of cultivation, the biomass of cells grown in media with glucose and 0.4 and 50 mM phosphate differed little at 1.55 and 1.66 g/L, respectively. Thus, in cultivation medium with glucose, the content of phosphate of 0.4 mM was sufficient for normal development of this strain, with the cells consuming almost all of the phosphate from the medium. Thus, 39 h after cultivation, the phosphate concentration was only 0.008 mM on average. A decrease in the phosphate concentration down to 0.1 mM lead only to a limitation of culture growth (Fig. 1).

With naphthalene as a carbon source, suppression of the culture growth was observed at a decrease in the phosphate concentration, down to 0.4 mM. After 64 h of cultivation, the amount of biomass in the media containing 0.4 and 50 mM of phosphate was 0.76 and 1.35 g/L, respectively. After 93 h of cultivation (the beginning of the stationary phase of growth) in medium with 0.4 mM of phosphate, the amount of protein in the biomass decreased by a factor of 3 (from 9.92 down to 3.36 mg/g of the biomass) as compared to the logarithmic phase of growth. The medium with naphthalene and 50 mM of phosphate had similar amounts of the protein in the stationary and logarithmic phases of growth, which is evidence of normal functioning of cells, in contrast to the culture grown in the medium with 0.04 M phosphate (Table 1). The phosphate content decreased to 0.12 mM 64 h after cultivation in the medium with naphthalene and its initial concentration of 0.4 mM.

Upon the cleavage of naphthalene, the formation of salicylic acid takes place, which is then oxidized to catechol. Catechol is broken down by two alternative pathways: the meta pathway, with the formation of acetylaldehyde and pyruvate, and the ortho pathway, with the formation of succinate and acetate [17, 18]. Much more rarely, gentisic acid is formed when salicylic acid is oxidized [19, 20] (Fig. 2). During the decomposition of naphthalene, phosphorylation of substrates does not occur; thus, an increase in the phosphate demand, when compared to glucose, could not be accounted for by features of the metabolic pathways of degradation.

It could be suggested that phosphate deficiency in the medium could affect the activity of key enzymes of biodegradation of naphthalene. To determine their activity, the *P. putida* BS 3701 strain was grown in Evans medium with 0.4 or 50 mM phosphate and naphthalene as the only carbon source.

The bacterium of the *P. putida* BS 3701 strain has a complete set of genes encoding enzymes involved in naphthalene biodegradation, including two genes encoding salicylate hydroxylase [22]. The activity of the key enzymes of naphthalene biodegradation (naphthalene dioxygenase, salicylate hydroxylase, catechol 1,2- and catechol 2,3-dioxygenase) was determined in logarithmic (45 h) and stationary (93 h) phase cultures. The results are given in Table 1. It was found that the specific activity of naphthalene dioxygenase and salicylate hydroxylase in the logarithmic phase culture of *P. putida* BS3701 in the medium with 0.4 mM phosphate was lower than that in the medium with 50 mM phosphate. In stationary phase cultures in the medium with 0.4 mM phosphate, the activity of these enzymes was absent. It should be noted that a

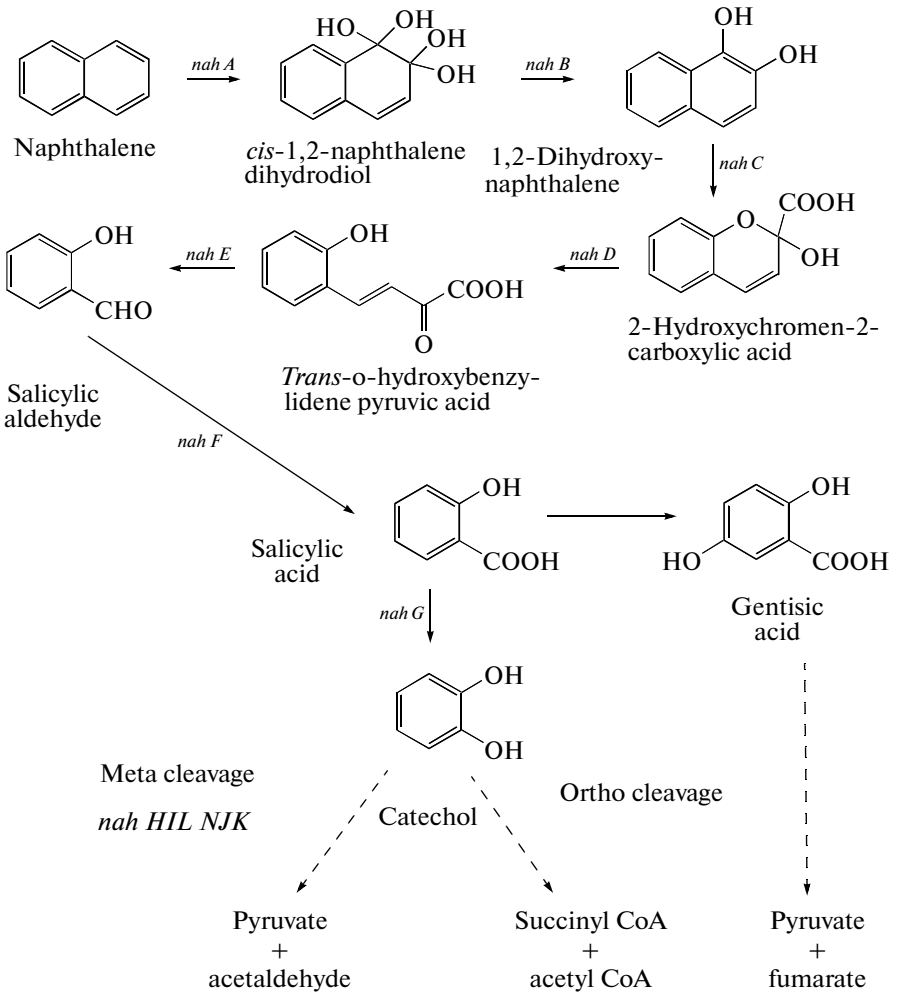

Fig. 2. Biochemical naphthalene degradation pathways [21].

higher activity of catechol 2,3-dioxygenase was observed in the logarithmic phase culture of *P. putida* BS3701 in the medium with 0.4 mM phosphate than in the medium with 50 mM phosphate (Table 1).

The genes of the enzymes of naphthalene biodegradation, which are localized in the NAH7 plasmid, are organized in two operons. The upper pathway of naphthalene oxidation (the *nah1* operon) triggers the genes encoding the cleavage of naphthalene to salicylate. The lower pathway of naphthalene oxidation (the *nah2* operon) is controlled by the genes encoding the synthesis of salicylate oxidation enzymes through meta cleavage of catechol to acetaldehyde and pyruvate. The expression of both operons is coordinately induced in the presence of salicylate [23]. The two

Table 1. Specific activity of enzymes of naphthalene biodegradation of the *P. putida* BS 3701 strain in Evans medium with naphthalene at different phosphate concentrations

Phosphate concentration in medium, mM	Cultivation time, h	Protein, mg/g biomass	Specific activity of enzymes, μM/min mg of protein;		
			NO*	SH**	C2,3O***
0.4	45	9.92	0.015 ± 0.002	0.029 ± 0.004	0.206 ± 0.003
	93	3.36	0	0	0
50	45	10.06	0.060 ± 0.001	0.070 ± 0.001	0.037 ± 0.005
	93	10.02	0.016 ± 0.003	0.143 ± 0.006	0.016 ± 0.001

* Naphthalene dioxygenase, ** salicylate hydroxylase, and *** catechol 2,3-dioxygenase.

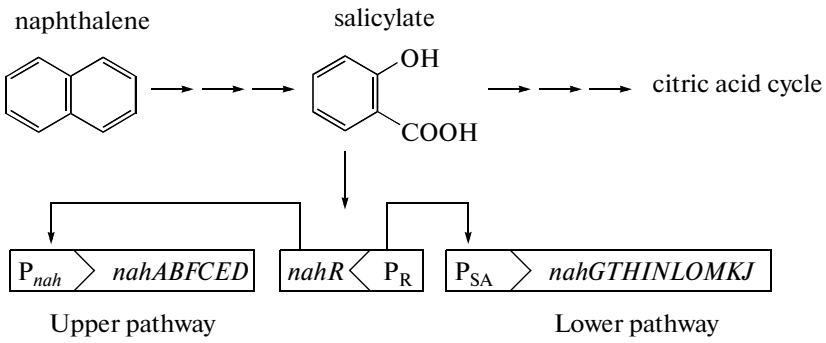

Fig. 3. Scheme of organization and regulation of expression of the *nah* genes of the NAH7 plasmid. Arrows indicate the direction of transcription [22].

operons are separated by a region (7 tbp) containing the *nah*R gene necessary for expression of the both operons [21, 24, 25]. The *nah*R gene is constitutively transcribed. The product of the *nah*R gene, the NahR protein interacting with salicylate, activates both *nah* operons. In a number of naphthalene–degrading bacterial strains, catechol oxidation occurs by the ortho pathway or simultaneously by the both ortho and meta pathways. The activity of catechol dioxygenases depends on the growth phase, condition of the cells, temperature, and other factors [26]. It could be suggested that the expression of catechol 2,3-dioxygenase in the logarithmic phase culture of *P. putida* BS 3701 in the medium with 0.4 mM phosphate was induced by the salicylate from the *nah2* operon of the lower pathway of naphthalene oxidation (Fig. 3).

Since the activity of all the enzymes was determined in 50 mM phosphate buffer, it could be concluded that phosphate did not significantly affect their activity observed in vivo. A change in the phosphate concentration in the cultivation medium seems to affect the expression of the enzymes of naphthalene degradation.

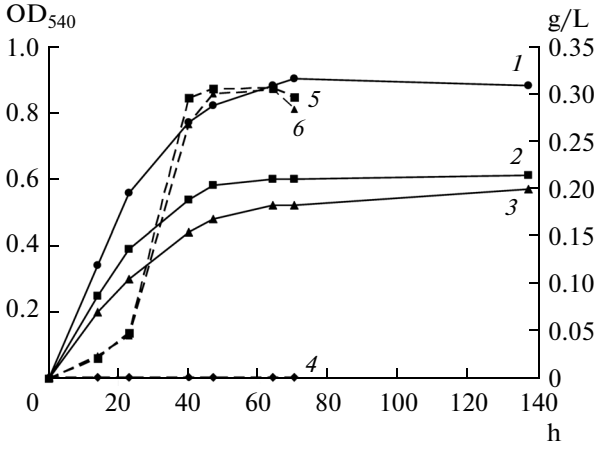

Fig. 4. Effect of different phosphate concentrations on biomass and salicylate accumulation in growth medium of *P. putida* BS 3701 with naphthalene. (*1*), (*2*), (*3*) accumulation of the biomass of *P. putida* BS 3701 in the medium containing 50, 0.4, and 0.1 mM phosphate, respectively; (*4*), (*5*), (*6*) salicylate accumulation in the medium with 50, 0.4, and 0.1 mM phosphate, respectively.

Since the activity of both naphthalene dioxygenase and salicylate hydroxylase was found to be from two to four times lower in the medium with 0.4 mM phosphate than in the medium with 50 mM phosphate, it was necessary to determine the salicylate concentration of one of intermediate products of naphthalene degradation in cultivation of *P. putida* on media with different contents of phosphate (Fig. 4). No accumulation of salicylate was observed in the culture medium with 50 mM phosphate. Salicylate was observed in the logarithmic phase culture in the medium with 0.1 and 0.4 mM phosphate. In both cases, up to 0.3 g/L of salicylate were accumulated by 39 h of the growth. This was in accordance with the results of the determination of the activity of salicylate hydroxylase, the first enzyme of the lower pathway (Table 1). The accumulation of this toxic metabolite seems to stop culture growth, causes cell death, and decreases the protein content.

Thus, determination of the enzyme activity and salicylate accumulation (two independent approaches) suggested that *P. putida* had impaired regulation of the expression of the genes of the upper and lower pathways of naphthalene oxidation at a low phosphate concentration in the medium.

Polyphosphates are able to regulate triggering of the genetic programs in some bacteria, including stimulation of the expression of the genes of the SOS pathway [2]. The content of orthophosphate, short-chain acid-soluble polyphosphates, and longer-chain acid-insoluble polyphosphates was determined in the stationary phase culture of *P. putida* BS 3701 in the medium with glucose or naphthalene and phosphate concentration of 50 mM. Cells of the *P. putida* BS 3701 strain were described by a low content of short-chain polyphosphates, which was comparable with the amount of orthophosphate, whereas the content of long-chain polyphosphates were several times higher (Table 2).

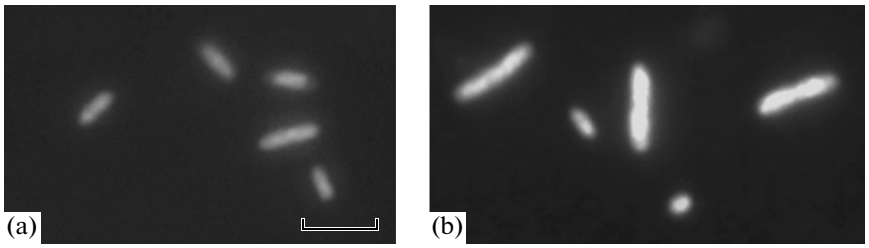

Fig. 5. Microphotographs of fluorescent cells of the *P. putida* BS 3701 stained with DAPI. (a) cells on medium with glucose and 50 mM phosphate at the logarithmic phase of growth, (b) bright cells grown on medium with naphthalene and 50 mM phosphate at the stationary phase of growth. Scale is 5 μm.

It was shown that the number of cells per biomass unit in cultivation on the medium with naphthalene is significantly smaller than that on the medium with glucose (Table 2). It should be noted that the products of naphthalene biodegradation, biosurfactants and polysaccharides, could be present in the biomass of cells grown on the medium with naphthalene [27]. Since these compounds could increase the mass of the biomass, the polyphosphate content was calculated per cell. Comparison of the content of P and polyphosphates in cells of *P. putida* BS 3701 demonstrated that, in the medium with naphthalene, the culture at the beginning of the stationary phase had a significantly greater amount of both orthophosphate and polyphosphates of both accumulated fractions as compared to these indices on the medium with glucose. Under phosphate deficiency in the medium, the biomass had no orthophosphate and short-chain acid-soluble polyphosphates (the data are not given), and the amount of polyphosphates accumulated was six times less than that in the medium with 50 mM phosphate. Since the phosphate concentration limiting the growth in the medium with naphthalene was higher than that in the medium with glucose, it could be suggested that polyphosphates are necessary for successful biodegradation of naphthalene by *P. putida*.

To determine the localization of polyphosphates in living cells, fluorescence microscopy with DAPI staining was used (Fig. 5). *P. putida* BS 3701 cells were grown to the beginning of the stationary phase in Evans medium with naphthalene or glucose and 50 mM phosphate. In the medium with glucose, most cells had green fluorescence, which is typical for a mixed spectrum of DAPI–DNA and DAPI–polyphosphates. Relatively short-chain polyphosphates appear to be accumulated in the medium with glucose. It is known that a shift of the spectrum of DAPI-polyphosphate complexes into the orange range is more typical for long-chain polyphosphates [2]. Cells grown in the medium with naphthalene had the fluorescence intensity in the orange region of the increased spectrum (Fig. 5, bright cells), since a significant amount of cells with the cell wall and individual granules fluorescing appeared in the population. The data of fluorescence microscopy were in accordance with the data obtained by studying the extraction products (Table 2). This made it possible to conclude that not only the number but also the degree of polymerization of polyphosphates increased in cultivation in the medium with naphthalene. These data do not contradict the data obtained earlier on the involvement of polyphosphates in the degradation of foreign compounds by bacteria [4, 5].

Table 2. Orthophosphate and polyphosphate content in *P. putida* BS 3701 strain cells at the stationary phase of growth in medium with 50 mM phosphate and glucose or naphthalene

Parameters	Culture medium	
	glucose	naphthalene
Biomass, g/L	1.425	1.285
Number of cells/mL of medium	2×10^8	3×10^7
Number of cells/g of biomass	1.4×10^{11}	1.6×10^{10}
Content of mineral phosphoric compounds, μmol P/cell $\times 10^{-11}$		
Orthophosphate	10	71
Acid-soluble polyphosphates	6	38
Acid-insoluble polyphosphates	53	160
Total content of polyphosphates	59	198

Thus, it has been shown that phosphate is a limiting factor of naphthalene degradation by *P. putida* BS 3701, which may be attributable to cell demand in the accumulation of inorganic polyphosphates. The mechanism of involvement of these polymers in the regulation of cell adaptation to naphthalene consumption requires further research. These compounds might be involved in the regulation of the expression of some genes encoding degradation enzymes, for example, salicylate hydroxylase. These polymers are known to be involved in the regulation of expression of SOS genes [1, 3]. It is possible that an increase in the polyphosphate content in a cell wall results in an increase in the resistance of the cell to biodegradation products. In the presence of toxicants, when a carbon source is sufficient for the microorganism growth, polyphosphates play the role of a shunt factor, providing energy storage. Such an accumulation of polyphosphates is known in yeasts cultivated in the presence of toxic concentrations of heavy metals [14]. It is possible that a similar shunt role for polyphosphates could exist in bacteria during the degradation of foreign compounds.

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