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EXPERIMENTAL ARTICLES

Microbial Degradation of Organophosphonates by Soil Bacteria

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Abstract—Bacteria that can utilize glyphosate (GP) or methylphosphonic acid (MPA) as a sole phosphorus source have been isolated from soil samples polluted with organophosphonates (OP). No matter which of these compounds was predominant in the native habitat of the strains, all of them utilized methylphosphonate. Some of the strains isolated from GP-polluted soil could utilize both phosphorus sources. Strains growing on glyphosate only were not isolated. The isolates retained high destructive activity after long-term storage of cells in lyophilized state, freezing to –20°C, and maintenance on various media under mineral oil. When phosphorus-starved cells (with 2% phosphorus) were used as inoculum, the efficiency of OP biodegradation significantly increased (1.5-fold).

Key words: organophosphonates, glyphosate, methylphosphonic acid, biodegradation, *Achromobacter* sp., *Ochrobactrum anthropi*.

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Intensive development of the chemical industry and application of various chemicals in human economic activity is generally associated with environmental pollution, which poses serious ecological problems. One of the classes of hazardous pollutants is organophosphates (OP), which are components of herbicides, insecticides, antibiotics, flame arresters, corrosion inhibitors, and are used as chelating additives to detergents [1]. Besides, detoxification of chemical warfare agents (sarin, soman, VX) results in formation of stable alkyl-phosphonic acids [2]. The properties of OP are determined largely by a covalent C–P bond, which is resistant to chemical hydrolysis, thermal damage, and photolysis [3]. Nevertheless, this bond can be broken by enzymatic systems of microorganisms that utilize the released phosphorus for biosynthesis of cell components. Many bacteria of the genera *Escherichia, Pseudomonas, Agrobacterium, Klebsiella, Arthrobacter, Bacillus*, and *Rhizobium*, as well as ascomycetes and basidial fungi, were found to biodegrade compounds with a C–P bond [3–5].

OP may have a direct activated (amino- and acetylphosphonates) or inactivated (alkyl phosphonates) C–P bond, which determines the multiple pathways of their catabolism [3, 6, 7]. OP with the activated C–P bond include glyphosate (GP), i.e., N-phosphonomethylglycine, which is an active ingredient of many herbicides. A representative of OP with the inactivated and more stable bond is methylphosphonic acid (MPA), an intermediate of organic synthesis and a product of hydrolysis of chemical warfare agents (sarin, soman, and VX gas).

OP-degrading microorganisms provide a basis for development of biopreparations for environmental cleanup technologies. The efficiency of such biotechnologies depends on a thorough investigation of destructive properties of microorganisms, optimization of degradation processes, and maintenance of stability of biopreparations.

The goal of the present work was to isolate and select bacterial strains degrading GP and MPA, to study the conditions of storage and maintenance of selected strains, and to optimize the process of OP degradation.

MATERIALS AND METHODS

Microorganisms. OP-degrading strains were isolated from soil samples taken from the fields of Krasnodar krai and treated with a GP-containing Roundup herbicide for a long time as well as from chemical plant territory polluted with alkyl phosphonates (Saratov oblast). Enrichment cultures were obtained by two methods:

(1) Herbicide-polluted soil was composted at 28–30°C under periodic stirring and moistening with a mineral medium containing GP (2.4 mg/m soil) as a component of the GroundBio herbicide for 3 months, inoculated into a liquid MS1 medium with 10 g/l of glutamate and 0.5 g/l of GP, and incubated on a shaker with sequential transfers twice a week, 20 passages in all. Selected associations were inoculated on a agarized LB medium containing the following (g/l): bacto pep-

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tone, 10; yeast extract, 5; NaCl, 5; pH 7.0; isolated pure cultures were different in morphological character: colony type, cell size, and mobility.

(2) A glass column (30 cm in height, 5 cm in diameter) was filled with OP-polluted soil, through which MPA solution was passed weekly for 3 months at the concentration increasing from 0.05 to 0.5 g/l. Soil samples taken from different depths were suspended in a mineral medium and appropriate dilutions were plated on agarized MS1 medium with MPA. All grown colonies were washed off, and the resulting mixed culture was used for isolation of pure cultures on rich agarized LB medium.

The effect of storage conditions and phosphorus starvation on destructive activity of the cells was studied in 3 strains: *Achromobacte*r sp. MPS 12 (destructor of MPA), *Achromobacter xylosoxydans* GPK 1, and *Ochrobactrum anthropi* GPK 3 (destructors of GP and MPA). Taxonomic affiliation of these strains was defined by analyzing the primary nucleotide sequences of their 16S rRNA genes using BLAST software [8]. Total bacterial DNA was isolated according to [9]. DNA concentration was measured in a TKO-100 fluorimeter (Hoefer Scientific Instruments, United States) with Hoechst 33258 dye (Bio-Rad, USA) according to the manufacturer's protocol.

Polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9700 cycler (Applied Biosystems, United States). The reaction was performed under standard conditions, with deoxyribonucleotide triphosphates in the final concentration of 200 μM, 1.5 mM $MgCl_2$, and, in some cases, 5% dimethyl sulfoxide (DMSO) (Sigma, United States). Oligonucleotide primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGHTACCTTGTTACGACTT-3') were used in the work. DNA electrophoresis was performed in 0.8% agarose in 0.5× TBE (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) according to the standard procedure [9]. DNA was visualized by gel staining in ethidium bromide solution. DNA was purified from the gel using Qiaex II Agarose Gel Extraction System (Qiagen GmbH, Germany) according to the manufacturer's protocol. DNA nucleotide sequence was determined by CEQ™ 2000XL DNA Analysis System using the DTCS (Dye Terminator Cycle Sequencing Chemistry) kit of reagents (Beckman, United States) according to the manufacturer's protocol. DNA amount in the reaction was 60–100 ng.

Medium and cultivation conditions. The bacteria were cultivated in a mineral MS1 medium containing (g/l): NH_4Cl, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; K_2SO_4, 0.5; and trace elements (mg/l): $FeSO_4 \cdot 7H_2O$, 2.5; $CaCl_2 \cdot 6H_2O$, 10.0; $CuSO_4 \cdot 5H_2O$, 2.0; H_3BO_3, 0.06; $ZnSO_4 \cdot 7H_2O$, 20.0; $MnSO_4 \cdot H_2O$, 1.0; $NiCl_2 \cdot 6H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.3. All salts were of "particularly high purity" or "chemical purity" grade. Sodium glutamate (Difco, United States) was used as the carbon source (10 g/l). The same amino acid could serve as a nitrogen source, along with NH_4Cl. MPA, 0.3 g/l (Aldrich, United States), or GP, 0.5 g/l, as a component of GroundBio herbicide (Monsanto, United States) was used as a phosphorus source.

The inoculum was grown for 3 days on agarized selective MS1 medium with GP or MPA; cells were washed off with liquid medium without a phosphorus source, collected into a sterile flask and, depending on experimental purposes, used for inoculation without starvation or after incubation on a shaker for 48 h under phosphorus starvation. In the latter case, 10 g/l of glutamate was added to the medium. Microorganisms were cultivated in 750-ml flasks with 100 ml of liquid MS1 medium on a shaker (180–200 rpm) at 28–30°C. The pH value of the medium during cultivation was maintained at 6.5–7.5 by adding sterile 20% H_2SO_4 solution.

Control of microbial growth and destructive activity. Growth was controlled by the change of optical density using a Specol 21 spectrophotometer at 560 nm (OD_{560}) and recalculated for dry biomass weight with coefficient 0.5 (g/U OD). Destructive activity of the strains (Q) was calculated as a ratio of utilized OP to the amount of biomass (mg_{OP}/g dry cells). The rate of survival for lyophilized and frozen cells was determined as a change of CFU numbers obtained by plating dilutions of the cell suspension on agarized LB medium 24 h after the procedure and after 3 and 6 months of storage.

Analytical methods. Concentration of inorganic phosphorus was determined spectrophotometrically by formation of a complex of phosphomolibdate and malachite green in an acidic medium [10]. Total phosphorus was determined by the same method after organophosphonate hydrolysis with ammonium persulfate [4]. The content of MPA and GP in the culture liquid was calculated as the difference between total and inorganic phosphorus by introducing respective conversion rates for each compound. The presence of glutamate in the medium was controlled by TLC on Silufol plates (LaChema, Czech Republic) in the system of *n*-butanol–acetic acid–water (12 : 3 : 5) followed by development with 0.5% ninhydrin solution in acetone. Total phosphorus in the cells was determined with ammonium molybdate after the biomass burning with perchloric acid [11].

RESULTS AND DISCUSSION

More than 40 strains were isolated from enrichment cultures obtained from OP-polluted soil under selective GP or MPA pressure, and their ability for growth under batch cultivation in liquid MS1 medium with these phosphonates as sole phosphorus sources was tested. Selection criteria were the following indices (the lower limit): biomass in the stationary growth phase in the media with MPA (2.0 g/l) and GP (1.0 g/l) and maximal specific growth rate, 0.04–0.05 h^{-1}. The isolates

Table 1. Growth of OP-degrading strains in the media with GP and MPA

Strain	Phosphorus source in enrichment culture	Growth in OP-containing liquid medium				Phosphorus source for maintenance at storage
		MPA		GP		
		Biomass, g/l	$\mu\ h^{-1}$	Biomass, g/l	$\mu\ h^{-1}$	
GPK 1	GP	2.3	0.08	1.5	0.06	GP
GPK 2	GP	2.1	0.07	1.8	0.04	GP
GPK 3	GP	2.7	0.16	3.3	0.10	GP
MGPK 4	GP	2.2	0.04	1.2	0.05	GP
MPK 5	GP	1.9	0.07	Weak		MPA
MPK 6	GP	1.9	0.04	Weak		MPA
MPK 7	GP	2.5	0.1	Weak		MPA
MPK 8	GP	2.1	0.08	Weak		MPA
MPS 9	MPA	2.2	0.07	Weak		MPA
MPS 10	MPA	2.3	0.08	Weak		MPA
MPS 11	MPA	2.2	0.12	Weak		MPA
MPS 12	MPA	2.4	0.16	Weak		MPA

Note: "Weak" is weak growth; biomass does not exceed 0.2 g/l.

obtained from the enrichment culture with MPA grew in liquid medium with this phosphorus source only; in the medium with GP, their biomass did not exceed 0.2 g/l. Some of the isolates obtained from the enrichment culture with GP grew equally well in liquid media with GP and MPA; other isolates grew well only in the liquid medium with MPA. Strains that utilized GP as a carbon or nitrogen source, or MPA as a carbon source, were not isolated.

Twelve of the most active OP-degrading strains were chosen (Table 1) and maintained on an agarized selective MS1 medium with the OP degraded by respective strains. It is known that physiological properties of the cultures may change significantly after microorganisms had been isolated from its native habitat and maintained on selective media in collections. Therefore, the choice of conditions for long-term storage of the strains without the loss of destructive activity, as well as determination of the survival rate of stored cells, are extremely important, particularly in view of their application in the technologies of bioremediation of polluted soils. The work was performed with *Achromobacter* sp. MPS 12 (destructor of MPA) and *A. xylosoxydans* GPK 1 (destructor of GP and MPA).

The change of destructive activity was tested after 6 months of storage in a lyophilized state, with freezing to – 20°C, and under sterile mineral oil at 8°C in semiliquid agar and on slanted agarized mineral MS1 medium with MPA. The choice of MPA for the growth of biomass for subsequent storage was due to the fact that all of the isolated strains utilized this phosphonate as a phosphorus source. The cultures maintained by monthly transfers in MS1 mineral medium with MPA and GP and stored at 8°C were used as controls. After the above period of storage, the bacteria were plated on a fresh agarized MS1 medium with MPA (both strains) or with GP (*A. xylosoxydans* GPK 1). The cells were transferred to a medium with no phosphorus source; the suspension was incubated for 24 h to achieve phosphorus starvation and then used to inoculate the liquid medium with the same phosphorus source that was used for the agarized medium. The culture was grown until transition to the stationary phase; the biomass and utilized OP were assayed.

Experimental results showed that both strains retained the ability to break the C–P bond of OP and to utilize phosphorus for the biosynthesis of cell components. The growth of biomass was limited by the amount of carbon, and its value for each culture in all experimental variants was comparable with the control (without preservation): 2.3–2.7 g/l (data not shown). Destructive activity was still high after 6 months of storage under all of the conditions described above and comparable with the activity under maintenance of the cultures by monthly transfers without preservation. The method of long-term storage under mineral oil on agarized mineral MS1 medium with OP provided the highest efficiency of OP utilization by the studied strains (Table 2). This method was also used for the strain *Ochrobactrum anthropi* GPK 3, subsequently selected as the most active GP destructor (40–80 mg GP/g biomass) and, moreover, growing on this phosphorus source with the maximal specific rate of 0.1–0.12 h^{-1}. The results confirmed that degradation efficiency of the strain stored on an agarized medium with GP under mineral oil also remained at the same level as in the control (75 and 78 mg/g, respectively).

Table 2. Efficiency of OP utilization and survival of the cells of destructor strains after 6 months of storage under different conditions

Storage conditions	Medium*	*Achromobacter* sp. MPS 12		*Achromobacter xylosoxydans* GPK 1		
		Q_{MPA}**	Survival, %	Q_{MPA}****	Q_{GP}**	Survival, %
Control***		47.1		33.1	27.4	
Freezing at –20°C	1	38.9	65–69	29.9	20.1	71
	2	41.2	63–71	31.4	Ì.Ó.	74–80
Lyophilization	1	42.0	43–46	24.4	22.0	45–52
	2	38.2	48–51	21.4	Ì.Ó.	44–47
Under mineral oil	3	36.0	N.a.	30.1	20.3	N.a.
	4	48.6	N.a.	34.0	25.2	N.a.

Notes: N.a.: not assayed.

* 1, liquid MS1 medium with MPA; 2, agarized LB medium; 3, semiliquid agar; 4, agarized MS1 medium with MPA.

** Q_{MPA} and Q_{GP} are destructive activities, mg/g biomass.

*** Control is monthly transfers to agarized MS1 medium with MPA or GP at 8°C.

The rates of survival of lyophilized cells and cells frozen at –20°C were determined by CFU number counting 24 h after the procedure, and freezing was shown not to affect the cell titer; in the course of lyophilization, however, the quantity of living cells decreased by an order of magnitude. The change of the quantity of surviving cells after 6 months of storage is shown in Table 2.

The presented data indicate that survival of the cells of both strains depends on the method of preservation: it is much higher for freezing than for lyophilization and does not depend on growth medium composition (selective medium with MPA or rich LB medium). It should be noted that the decrease of the cell titer was most pronounced during the first 3 months and did not exceed 4–7% in the following period (data not shown).

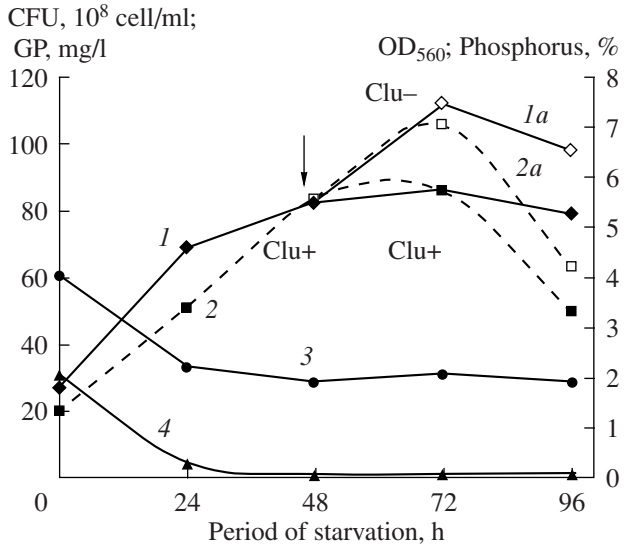

Fig 1. The change of characteristics of strain *Ochrobactrum anthropi* GPK 3 under phosphorus starvation: *1*, OD_{560}; *2*, CFU; *1a, 2a*, the same after additional introduction of GP; *3*, total phosphorus content in the cells; *4*, GP concentration in the medium. The arrow indicates the time of additional GP introduction (150 mg/l). "Glu+" and "Glu–" denote the presence and absence of glutamate in the medium.

The presence of easily assimilated phosphorus sources in the medium blocks the action of enzyme systems responsible for OP degradation in bacteria: C–P lyase, GP-oxidoreductase, and phosphonatase [1, 12]. Therefore, a necessary condition for increase of OP degradation efficiency in the bacteria is the absence of alternative phosphorus sources both in the medium and in the cells of inoculated material. This may be achieved through preliminary starvation of cell suspension under incubation with a carbon source but without a phosphorus source.

The experiments on phosphorus starvation were performed with the strains *Ochrobactrum anthropi* GPK 3 and *Achromobacter* sp. MPS 12. The cultures were grown on agarized MS1 medium with glutamate as a carbon source and GP or MPA; cells were washed off with a liquid mineral medium without OP, suspended until OD 2.0 followed by addition of glutamate (10 g/l), and incubated on a shaker for 96 h. The initial cell concentration in the starving suspension was experimentally determined to be optimal for the given conditions. At a higher initial density of suspension, the carbon source was rapidly depleted and double limitation by carbon and phosphorus occurred, which resulted in cell lysis; besides, the cells could suffer oxygen deficiency.

The data presented in Figure 1 for the strain *Ochrobactrum anthropi* GPK 3 grown in GP-containing medium show that this OP was completely consumed in 48 h in the presence of a carbon source, while the biomass increased 3.5–4.0 times both by the number of living cells (CFU) and by OD, whereas the content of total phosphorus in the cells decreased from 4.5 to 2.1–2.4%. Inorganic phosphorus was not detected in the

Table 3. The effect of duration of phosphorus starvation of inoculum on biomass accumulation and phosphorus source uptake at a growth of strain *O. anrthropi* GPK 3 in GP-containing medium

Duration of inoculum starvation, h	Biomass		GP utilization		
	g/l	CFU, 10^9 cells/ml	mg/l (%)	Destructive activity, mg/g biomass, %	Destructive activity, μg/10^9 cells, %
0	2.95	7720	146.6 (100)	49.7 (100)	19.0 (100)
24	2.85	7690	207.0 (141)	72.6 (146)	26.9 (141)
48	2.85	7280	223.6 (153)	78.4 (158)	30.7 (161)
72	2.95	8140	197.0 (134)	67.0 (135)	24.2 (127)
96	3.0	7155	146.0 (100)	48.6 (100)	20.4 (107)

medium. These results suggest that biomass growth in the presence of a carbon source stopped because of phosphorus deficiency resulting from depletion of the intracellular phosphorus reserves and residual GP in the medium. Apparently, the decrease of phosphorus content to 2% during starvation is the threshold concentration, below which the intracellular reserves cannot be used for the synthesis of cell components. This assumption was confirmed by the fact that additional introduction of GP into the medium after 48 h of incubation resulted in a renewal of growth, which stopped after 24 h, this time due to depletion of the carbon source (glutamate). With depletion of carbon or phosphorus sources, the number of living cells (CFU) abruptly decreased as a result of their lysis, whereas OD indices were less affected by these changes. Similar results were obtained under starvation of the cells of both strains grown in MPA-containing medium; i.e., the biomass increased 2.5–3.5 times (by OD and CFU values) after 24–48 h and the residual amount of MPA was completely utilized.

The second series of experiments was performed to study the change of physiological characteristics of the strains *O. anthropi* GPK 3 and *Achromobacter* sp. MPS 12 under batch cultivation in GP- or MPA-containing media, depending on the degree of phosphorus starvation of the inoculum. The results for the strain GPK 3 growing in GP-containing medium are presented in Table 3. In all variants, culture growth stopped due to complete depletion of the carbon source, because additional introduction of glutamate after transition into the stationary phase resulted in growth renewal (data not shown). The amount of grown biomass did not depend on the duration of inoculum starvation, while GP consumption and specific efficiency of its uptake for the whole period of cultivation increased by 40 to 60% when the medium was inoculated with cells that had starved for 24–48 h and contained half as much phosphorus as non-starved cells (Fig. 1). The same pattern was obtained for cultivation of the bacteria in MPA-containing medium: degradation efficiency of strains *O. anthropi* GPK 3 and *Achromobacter* sp. MPS 12 was 35 and 41 mg MPA/g, respectively, for inoculation of the medium with non-starved cells and 47 and 54 mg MPA/g, respectively, with the inoculum after starvation for 48 h.

In the third series of experiments, the change of destructive activity and total phosphorus content in the biomass were studied in the growth dynamics of strain *O. anthropi* GPK 3 in GP-containing medium inoculated with the cells exposed to phosphorus starvation for 48 h. During the lag phase, the content of total phosphorus in the cells increased from 2 to 5.1% and then decreased to 4.0–3.8% in the period from the beginning of active reproduction of cells to transition into the stationary phase (Fig. 2). At the same time, the intensity of GP uptake decreased from 1098 mg/g biomass in the lag phase to 53.6 mg/g in the period of active culture growth. It should be noted that GP uptake in the lag phase was 17–20 times higher than in the period of active culture growth, with either non-starved or

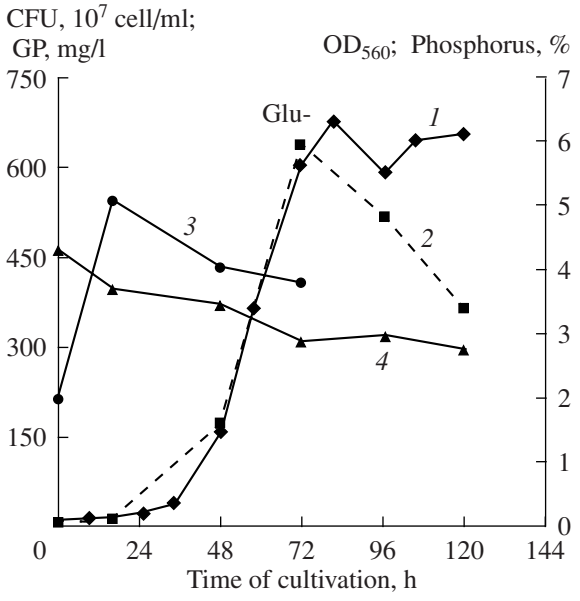

Fig. 2. The growth dynamics of strain *O. anthropi* GPK 3 in GP-containing medium inoculated with the cells starved by phosphorus for 48 h: *1*, OD_{560}; *2*, CFU; *3*, total phosphorus content in cells; *4*, GP concentration in the medium. "Glu–" denotes the absence of glutamate in the medium.

starved cells used as inoculum. The amount of utilized OP varied depending on the duration of preliminary starvation of the inoculum: 766 mg/g in the lag phase and 43.6 mg/g in the growing culture inoculated with non-starved cells, increasing to 1200 and 55 mg/g, respectively, for inoculation with the cells starved for 48–72 h. Such a pattern was also observed in the changes of specific efficiency of GP uptake per number of living cells (CFU). With the inoculum of higher degree of starvation (96 h), the amount of GP taken up from the medium decreased in the dynamics of culture growth.

Thus, no matter which OP (MPA or GP) predominated as the phosphorus source in the native habitat of the strains, all of them could utilize MPA as a phosphorus source and only some strains could utilize both substrates. Conditions of long-term storage of the cells were determined in order to provide maintenance of their destructive activity at a high level. The efficiency of OP biodegradation significantly increased for application of phosphorus-starved cells as inoculum containing 2% phosphorus in its biomass. The data obtained are a necessary stage in the study of OP biodegradation conditions for development of technologies for decontamination of soils polluted with these toxicants.

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