

## Degradation of Perfluorooctanyl Sulfonate by Strain *Pseudomonas plecoglossicida* 2.4-D

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**Abstract**—A bacterial strain has been isolated from soil contaminated by waste from petrochemical production. Based on its cultural-morphological, physiological-biochemical properties and an analysis of the nucleotide sequence of the 16S rRNA gene and phylogenetic analysis data, the strain was defined as *Pseudomonas plecoglossicida* 2.4-D. The unique ability of this bacterium to use perfluorooctanyl sulfonate (PFOS) as the only source of carbon and energy is shown. When cultivated in a liquid medium, the strain completely utilizes this substance in 6 days. It is proved that *P. plecoglossicida* 2.4-D transforms PFOS to perfluoroheptanoic (perfluorooctanoic) acid, while free fluorine ions are released into the medium. Upon introduction into the soil, the strain was capable of PFOS degradation by 75%. The strain *P. plecoglossicida* 2.4-D is recommended for environmental protection. It can be used in the development of biotechnologies for the transformation (utilization) of fluoroorganic compounds.

**Keywords:** perfluorinated compounds, *Pseudomonas*, degradation, transformation, perfluorooctanyl sulfonate, PFOS

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### INTRODUCTION

The modern growth of the chemical industry and organic synthesis has been accompanied by environmental pollution by a wide spectrum of xenobiotics detected in the components of natural geosystems; they are toxic, able to bioaccumulate, and resistant to degradation. Most of these pollutants are halogenated compounds, which are often detected in industrial effluents. The toxic properties of these compounds make it necessary to use microorganisms in their utilization. The most resistant of the compounds comprising industrial emissions into the environment are fluorine-containing compounds, particularly, perfluorocarboxylic acids—synthetic chemical compounds used in the industry of widely used fluoropolymers. They also include surfactants (SAAs), which possess a high chemical stability that makes them an ideal material for extensive use in the production of different materials for waterproof and stain resistant coatings in textiles and lubricants to firefighting foam.

Perfluorocarboxylic acids were included in the Addendum of the Stockholm Convention on Persistent Organic Pollutants, which recommended that measures be taken to minimize and, if possible, stop the production and use of these compounds [1].

In 2002 the biggest companies of the world—producers of fluorinated surfactants—stopped the production of products with perfluorooctane sulfonate (PFOS)

or its derivatives. The main reason to stop production was the negative effect on the environment. PFOS, which resistant to biodegradation, is now detected in most objects of the environment and living organisms. It is the most frequently detected pollutant of this class [2–5].

PFOS pollution makes the problem of utilization of these ecologically dangerous pollutants very topical. Their incorporation into the natural metabolism and energy exchange with the participation of microorganisms capable of mineralization can minimize their negative effect on the environment. There are currently only individual reports concerning bacteria that utilize perfluorocarboxylic acid and PFOS. They do not reveal characteristics of biodegradation [6, 7]; however, it is known that other types of organofluorine compounds, such as nitroaniline, fluoroacetate, fluorobenzene, perfluoro biphenyls, perfluorohexyl sulfonate, etc. [8–12], can undergo biodestruction.

The goal of this work is to study the taxonomic position and characteristics of a new PFOS-degrading strain extracted from soil polluted with waste from the petrochemical industry.

### EXPERIMENTAL

The object of study was a natural strain extracted from a sample of soil contaminated by waste from pet-

rochemical production in a factory area (Bashkortostan republic, Russia).

Cumulative and pure cultures were obtained with the use of Raymond's mineral medium containing PFOS as the only source of carbon (0.1 wt/vol %) [13] (g/L):  $\text{Na}_2\text{CO}_3$ —0.1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.2;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —0.02;  $\text{CaCl}_2$ —0.01;  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ —0.02;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ —1.0;  $\text{NaH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ —1.5;  $\text{NH}_4\text{Cl}$ —3.0. Culturing was performed at 28°C on a shaker at 160 rpm. The intensity of culture growth was estimated by the optical density of the cell suspension at a wavelength of 590 nm with a SF-56 spectrophotometer (Lomo, Russia).

The characterization of pure culture was performed on the basis of cultural-morphological and physiological-biochemical indicators by the generally accepted guides [14, 15].

Total DNA extraction was conducted in accordance with the method described in [16].

Amplification of the 16S rRNA gene was performed with universal primers [17]. Extraction and purification of the PCR products was conducted from low-melting agarose with a Wizard PCR Preps kit (Promega, United States) in accordance with the manufacturer's instructions.

Sequencing of the obtained PCR products of 16S rRNA gene was performed with a Big Dye Terminator v.3.1 kit (Applied Biosystems Inc., United States) with an ABI PRIZM 3730 automated DNA Sequencer (Applied Biosystems, Inc., United States) in accordance with the manufacturer's instructions.

The search for nucleotide sequences of 16S rRNA genes homologous to the corresponding sequences of the examined strain in the GenBank database was performed with the BLAST program (<http://www.ncbi.nlm.nih.gov/blast>) [18]. The dendrogram of the phylogenetic similarity was built in MEGA6 software [19] by the neighbor-joining method [20] with the Kimura model [21].

For the extraction and identification of products of PFOS biotransformation and the determination of its concentration in the environment, bacterial cells were separated from the medium by ultrafiltration with Viva-flow 50 (Sartorius AG, Germany). The obtained filtrate ( $\leq 3$  kDa) was analyzed by chromatography mass spectrometry on tandem a LCMS-IT-TOF chromatograph mass spectrometer (Shimadzu, Japan) with a system for the introduction of eluted ions, a quadrupole ion trap, and a time-of-flight detector. The mass spectra were registered in the mass interval  $m/z$  200–800 a.e.m. in negative-ion mode, and the voltage in the detector was 3.5 kV. A Shim-pak XR-ODS column ( $75 \times 2.0$  mm) (Shimadzu, Japan) in isocratic mode with a 56 : 44 solvent ratio of 5 mM ammonium acetate in water : acetonitril at a flow rate of 0.2 mL/min was used for chromatographic division. Detection of the structure of the obtained substances was performed on the basis of total

mass spectrometry data, based on degradation of the molecular ion and a comparison with the literature data.

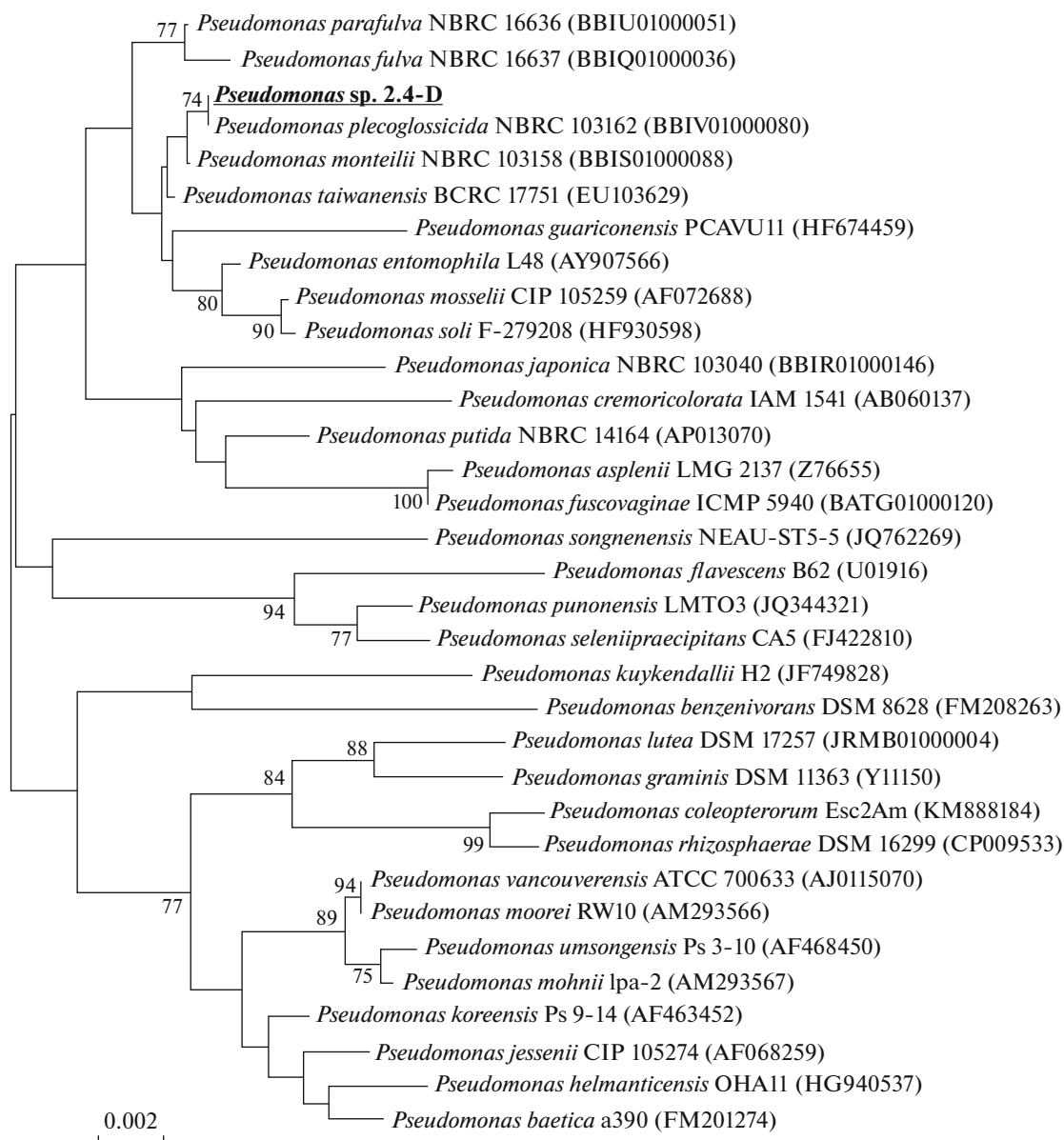
The concentration of fluoride ions in the media was measured with a fluoride-selective electrode with an ELIS-131F crystal membrane (Measuring Equipment, Russia)

The ability to degrade PFOS in soil was estimated under the conditions of a model experiment, in which 100 mL of cultured suspension of  $\text{OD}_{590} = 0.8$  (with 100 mL of tap water as the control) was introduced into the soil (per 1 kg), remixed with 0.5% PFOS, and left at room temperature. The soil humidity was maintained by the introduction every 0.5 months of 100 mL of tap water per 1 kg of soil with mixing. Soil samples (100 g each) were taken after 0.5, 1, 2, 3, and 6 months, and extraction with dichloromethane was conducted for 10 min. The extract was filtered through filter paper in a water bath at 50°C. The residual PFOS content was evaluated gravimetrically.

## RESULTS AND DISCUSSION

The cells of the studied 2.4-D strain are gram-negative moving bacteria with a diameter of 1.0  $\mu\text{m}$  and a length of 2.5–3.0  $\mu\text{m}$ . In culturing on MPA, the strain formed colonies of a white-cream colour that were round, convex, and 4–5 mm in diameter. The metabolism was respiratory; the 2.4-D strain was catalase-positive, synthesized oxidase, was unable to perform denitrification or hydrolysis of casein, gelatin, lecithin, and starch, did not synthesize lipolytic enzymes, did not grow on twin-80 medium, and tested positive for arginine dihydrolase. The optimal growth was at a temperature of 26–30°C, and the optimal pH was 6.8–7.2. Intensive growth was observed with an NaCl concentration of 0–5%, while growth appeared to be slow with a NaCl higher concentration, up to 10%.

The 2.4-D strain did not use the following as the sole source of carboxide: glucose, saccharose mannitol, fructose, sorbitol, inositol, maltose, arabinose, xylose, mannose, galactose, lactose, rhamnose, meso-inositol, starch, levan, potassium tartrate. Succinate, malate, citrate, 2-ketogluconate, ethanol, n-butanol, propylene glycol, L-leucine, L-lysine, L-valine, L-alanine, L-arginine, L-aspartate, L-histidine were used as a carboxide source. The cells of the strain synthesized fluorescent pigment. The gene sequence (1383 bp) for the extracted strain was later detected and deposited in the GenBank under the number KY593189. The gene codes 16S rRNA, which corresponds to sites 37 to 1419 according to *E. coli* nomenclature. The bacteria closest to the studied specimen were *Pseudomonas plecoglossicida* and *P. monteilii*; the level of similarity of the sequences of 2.4-D and *P. plecoglossicida* NBRC 103162, *P. monteilii* NBRC 103158 strains was 99.86 and 99.78% respectively. Comparative analysis of the nucleotide sequences of the 16S rRNA gene of the



**Fig. 1.** Phylogenetic position of the *P. plecoglossicida* 2.4-D strain in accordance with analysis of the nucleotide sequence of the 16S rRNA gene. The scale shows the evolutionary distance corresponding to two nucleotide substitutions for each 1000 nucleotides. The statistical significance of the order of branching defined by bootstrap analysis is shown in numbers. (Bootstrap values higher than 70% are shown).

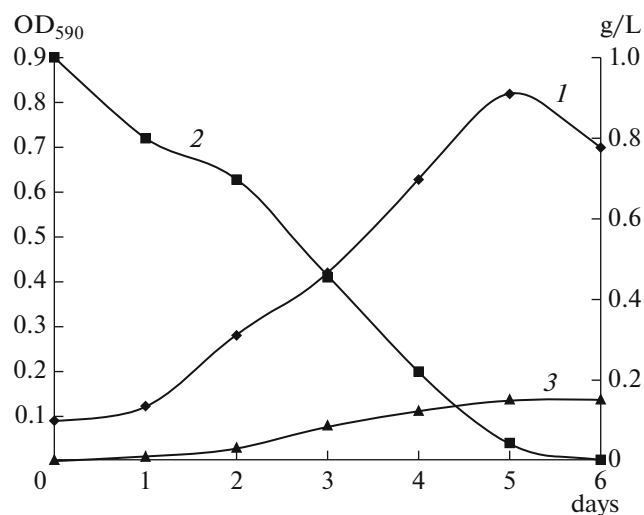
related pseudomonas species was conducted with the construction of a dendrogram to detect the phylogenetic position of the strain (Fig. 1). The obtained data made it possible to identify the studied strain as *Pseudomonas plecoglossicida* 2.4-D.

The *Pseudomonas plecoglossicida* 2.4-D strain actively degrades PFOS as a sole source of carboxide and energy in periodical culture (Fig. 2). Analysis of the dynamics of the decrease in the PFOS concentration in the *P. plecoglossicida* 2.4-D cultural medium showed that the culture adopted to substrate or performed preparatory metabolism in the first days

(decrease in the substrate concentration to less than 10%). Further linear growth of the substrate consumption of about 2% per day was observed. At the same time the optical density of the culture medium started to increase from the second day of cultivation, reaching its maximum at day 5 of cultivation.

It should be noted that an increase in the substrate concentration to 0.5% inhibits bacterial growth, and growth was blocked at concentration higher than 1.0%.

PFOS conversion was accompanied by the release into the medium of free fluoride ions. Their concentration reached 150 mg/L in the culture medium (CM);

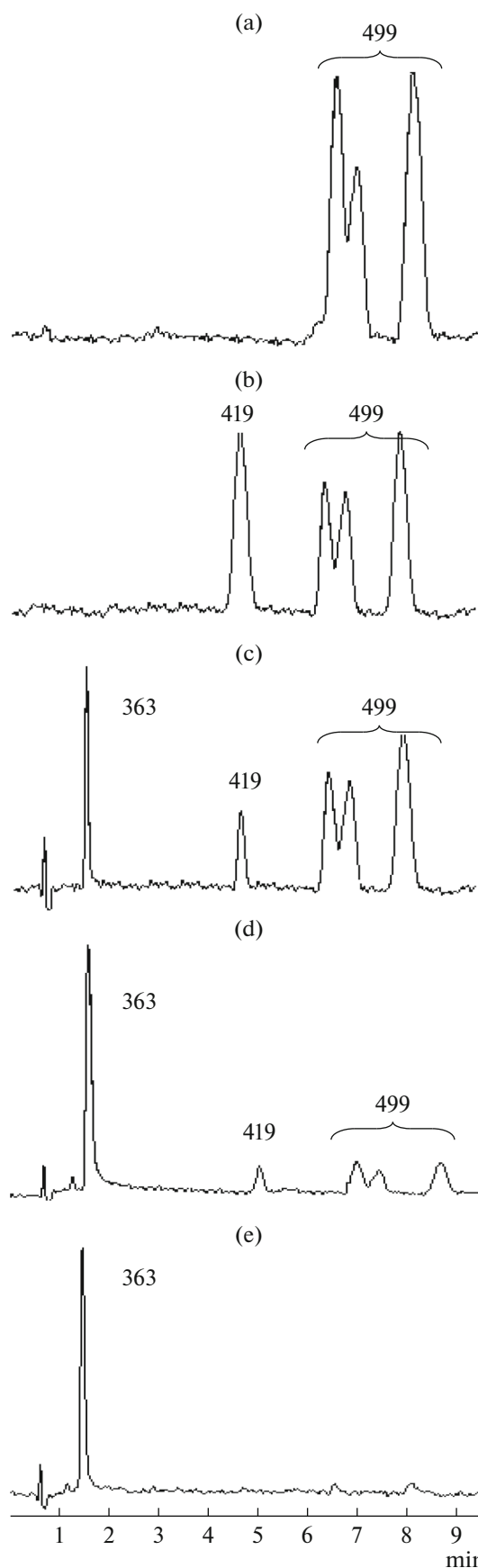


**Fig. 2.** Dependence of OD<sub>590</sub> values of the culture medium (1), PFOS concentrations (2), and free fluoride ions (3) from the time of *P. plecoglossicida* 2,4-D cultivation in periodical culture are shown.

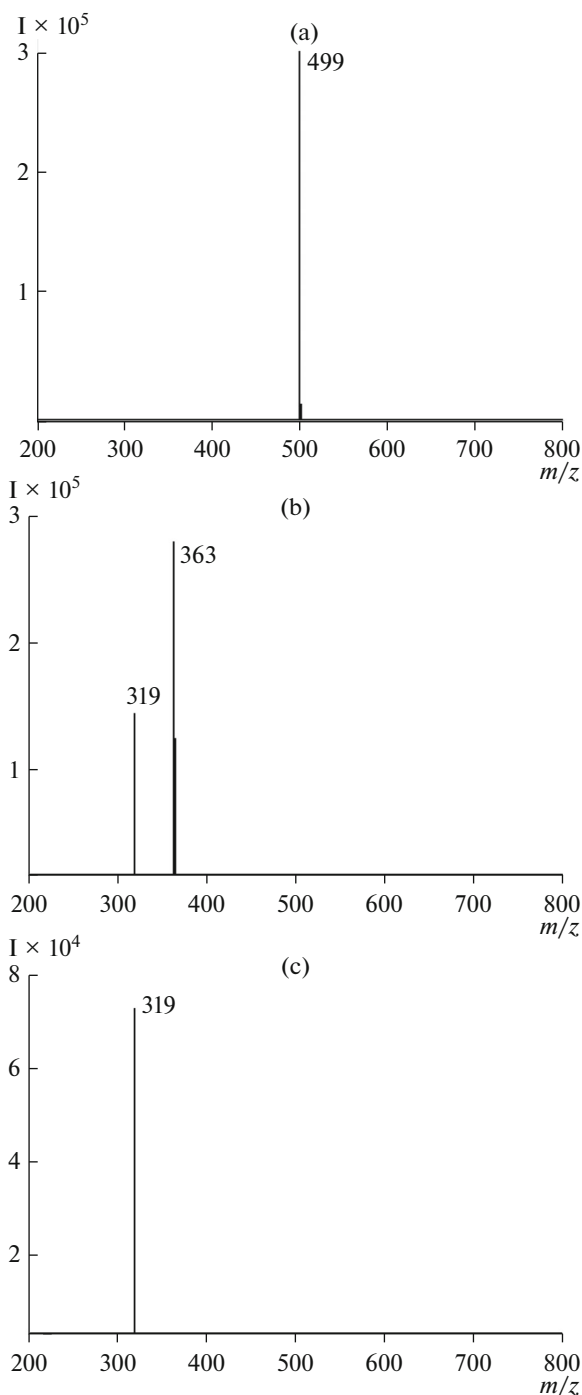
the beginning of the release correlated with the beginning of the linear decrease in the PFOS concentration.

The most preferable method for analysis of perfluorocarboxylic acids and anionic substances (including PFOS) is currently liquid chromatography–mass spectrometry.

As is known from the literature, perfluorinated organic acids are neutral and hardly biodegraded. A dissociated acid ion is usually observed in the process of chromatography–mass spectrometry of anionic perfluorinated compound. The transformation of PFOS (a molecular ion with an  $m/z$  of 499 amu, is presented in Fig. 3; the mass spectrum MS<sup>1</sup> is in Fig. 4a) for 6 days in conditions of periodical culture. After 1 day, the presence of a component with a molecular ion with an  $m/z$  of 419 amu is possible when performing oxygenogenic or monooxygenase removal of a sulfonate group from PFOS ( $m/z$  of 80 amu) in the form of sulfite, which can be metabolized in starvation conditions. This agrees with the supposition on the bacterial preparative metabolism for further defluorination. Further transformation (2–5 days) was accompanied by active growth of the culture and release of fluoride ions into the medium. After 3 days of cultivation in ultrafiltrate, a compound previously absent in the medium with a molecular ion equal to an  $m/z$  of 363 amu was found, along with components with  $m/z$  499 and 419 compounds (Fig. 3c). On the following day, the concentration of this component in CL increased, and

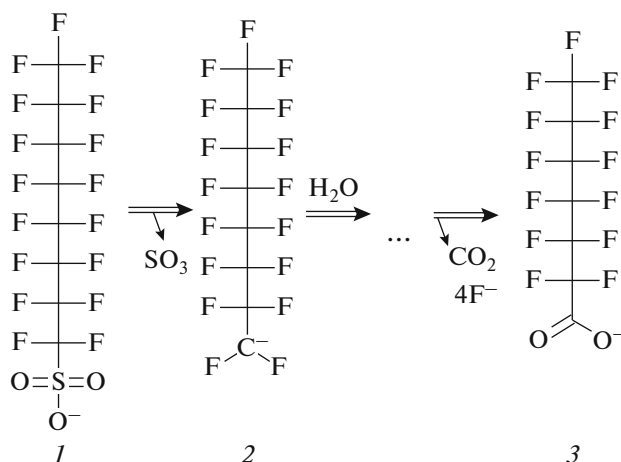


**Fig. 3.** Mass-chromatograms of *P. plecoglossicida* 2,4-D culture liquid ultrafiltrates after 0 (a), 2 (b), 3 (c), 4 (d), and 6 (e) days of cultivation in the periodical culture ( $m/z$ , amu are indicated above the peaks).

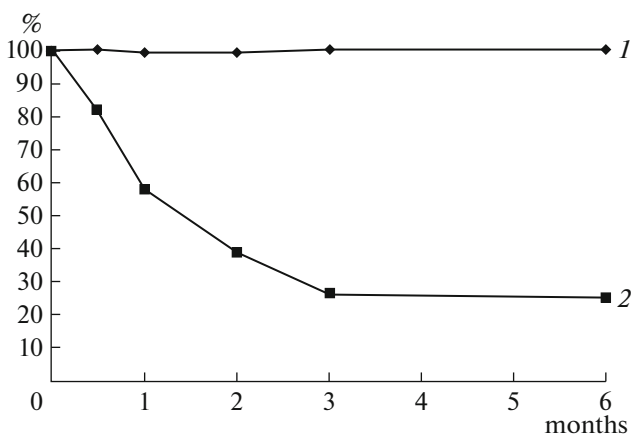


**Fig. 4.** MS<sup>1</sup> mass spectra of PFOS (molecular ion with an  $m/z$  of 499) (a) and MS<sup>1</sup> (b) MS<sup>2</sup> (c) component with an  $m/z$  of 363 amu from the *P. plecoglossicida* 2.4-D CL ultrafiltrate after 3 days of cultivation. Liquid Chromatography-Mass Spectrometer LCMS-IT-TOF (Shimadzu, Japan).

compounds of  $m/z$  499 and 419 amu were not identified 6 days after cultivation. According to the MS<sup>1</sup> and MS<sup>2</sup> mass spectra, the compound with an  $m/z$  equal to 363 amu was identified as perfluoroheptane (perfluoroenanth) acid. This acid commonly has a precursor ion



**Fig. 5.** Suggested scheme of bacterial PFOS destruction by *P. plecoglossicida* 2.4-D strain: 1—PFOS ( $m/z$  of 499 amu), 2—perfluorooctane ( $m/z$  of 419 amu), 3—perfluorooctanoic acid ( $m/z$  of 363 amu).



**Fig. 6.** Residual PFOS quantity in the soil after introduction of *P. plecoglossicida* 2.4-D strain (1). Control (2) without the induction of microorganisms.

with an  $m/z$  of 363 in the MS<sup>1</sup> mass spectrum (Fig. 4b); its fragmentation results in an ion product with an  $m/z$  of 319 in the MS<sup>2</sup> mass spectrum (Fig. 4c).

Thus, perfluoroheptane acid, which was identified by a dissociated acidic ion, was found in the culture medium at the end of cultivation. The results allowed the supposition of the following scheme of PFOS destruction (Fig. 5), in which four fluoride ions are released into the medium, corresponding to their concentration (152 mg/L) and correlating with the obtained result (1520 mg/L) with an initial PFOS concentration in the medium of 1.0 g/L.

The release of fluoride ions into the medium probably caused an inhibiting effect on the process of further destruction of intermediate fluoride compounds by the studied strain.

As follows from Fig. 6, in an experiment on the detoxification of soil polluted by PFOS, the bacteria

*P. plecoglossicida* 2,4-D showed the ability to actively transform the substance, decreasing its residual content to a level not exceeding 25% of the initial value. At the same time, the dynamics of the decrease in the concentration was linear for the first two months, while significant changes were not noted in the control.

An analysis of the literature data concerning the bacterial destruction of perfluorooctane sulfonic and perfluorooctane acids showed that the number of bacterial strains able to use these substances is extremely limited. *Pseudomonas aeruginosa* HJ4 [6] and the phylogenetically related strain *P. parafulva* YAB1 [7] were described, but no references concerning PFOS as a single source of carboxide and energy were found.

## CONCLUSIONS

Thus, as a result of the conducted studies on the basis of sum of cultural-morphologic, physiological, and biochemical signs and phylogenetic analysis data, the assignment of the 2,4-D strain to a species was conducted. It was detected that *Pseudomonas plecoglossicida* 2,4-D bacteria possessed a unique ability to use PFOS in water and soil as the sole source of carboxide and energy. The obtained data allow recommendation of this strain for use in the development of biotechnologies concerning the transformation of organofluorine compounds for the protection of the environment and are also a basis for the further studies of the adaptive-destructive potential of these bacteria.

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