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EXPERIMENTAL ARTICLES

Transformation of Low-Molecular Linear Caprolactam Oligomers by the Caprolactam-Degrading Bacterium *Pseudomonas putida* BS394(pBS268)

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Abstract—A biosensor based on the most active caprolactam-degrading strain *Pseudomonas putida* BS394(pBS268) was used in the study of aerobic degradation of linear caprolactam oligomers by bacterial cells. The changes in the respiratory activity of the strain depend quantitatively on caprolactam dimer concentration, making it possible to develop biosensors for detection of caprolactam oligomers in aqueous media. Based on mass spectrometry data, the scheme of transformation of linear caprolactam oligomers by the degrader strain *P. putida* BS394(pBS268) was proposed for the first time. It was found that oxidative transamination to respective dicarbonic acids may be one of the mechanisms of transformation of linear caprolactam oligomers. According to the scheme proposed, the ability of the caprolactam-degrading strain to transform linear oligomers results from the broad substrate specificities of two enzymes of the caprolactam degradation pathway: 2-oxoglutarate-6-aminohexanoate transaminase and 6-oxohexanoate dehydrogenase. Transformation of linear oligomers is genetically controlled by the CAP biodegradation plasmid pBS268.

Key words: biotransformation, linear caprolactam oligomers, CAP plasmid, transaminase activity, biosensors for caprolactam detection.

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Modern intensive industrial production leads to a considerable increase of environmental stress, which is evinced by significant contamination of the environment with toxic compounds. Among the most widespread pollutants in the contemporary world are a polymer based on 6-aminohexanoic acid and caprolactam (polycaproamide) and polymer materials (caprone, nylon-6, nylon). Extensive application of polycaproamide in combination with its low bioavailability poses the problem of utilizing outworn caprone products. Hence, microbial biotransformation of polymers and caprolactam oligomers (6-aminohexanoic acid oligomers) present in large amounts in the waste of polymer factories is quite an urgent matter.

The linear caprolactam oligomers and nylon are utilized by microorganisms of the genera *Achromobacter* [1–3], *Alcaligenes* [4], *Corynebacterium* [5, 6], *Flavobacterium* [7], *Pseudomonas* [8], and *Agromyces* [9].

To date, the biochemistry of oligomer degradation has been studied best for *Flavobacterium*. Degradation of caprolactam oligomers requires the presence of several specific enzymes: 6-aminohexanoate-cyclic dimer hydrolase, 6-aminohexanoate dimer hydrolase, and endogenous 6-aminohexanoate oligomer hydrolase; the genes encoding these enzymes are located on plasmids [10]. The cumulative activity of these three enzymes provides for transformation of the oligomer mixture to aminocapronoic acid.

Thus, biodegradation of caprolactam oligomers begins with the hydrolysis of amide bonds by specific hydrolases with the formation of 6-aminohexanoate, which is then sequentially transformed by the enzymes of the caprolactam catabolic pathway into adipic semialdehyde, adipate, and Krebs cycle intermediates [11]. The capacity of bacterial strains from the genus *Pseudomonas* for growth on ε-caprolactam and its intermediates was shown to be controlled by conjugative plasmids determining the degradation of ε-caprolactam at least to succinate [12]. It should be noted that no data are available on the existence of any other catabolic pathways for caprolactam and its oligomers.

It has been shown that bacteria may adapt to the presence of caprolactam oligomers, i.e., become able to biodegrade these xenobiotics. Several possible mechanisms of the onset of enzyme activities against synthetic substrates (including caprolactam oligomers) have been proposed: expansion of the substrate specificities of the enzymes present in a cell, activation of cryptic genes as a result of mutations, and alteration of regulatory mechanisms [5, 8]. The mechanisms of

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acquisition of the ability to degrade caprolactam oligomers have not been elucidated as yet.

The goal of this work was to study the ability of caprolactam-degrading bacteria to transform linear caprolactam oligomers by the example of a *Pseudomonas* strain.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The plasmid-bearing strain *Pseudomonas putida* BS394(pBS268) ($Cys^-Rif^R Sm^R$) and the plasmid-free strain *Pseudomonas putida* BS394 used in the present work were obtained from the collection of the Laboratory of Plasmid Biology, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The bacteria were grown in a liquid synthetic Evans medium [13] on a BIOSAN ES-20/60 orbital shaking incubator (Latvia) at 28°C and 180 rpm. The concentration of carbon sources (caprolactam for the plasmid-bearing strain and glucose for the plasmid-free strain) was 0.1–0.2%; the concentration of cysteine required for the growth of auxotrophic strains was 50 μg/ml. Agarized media were obtained by adding Difco agar (1.5%).

Microbial immobilization. Bacterial cells in the logarithmic growth phase were precipitated by centrifugation (5000 *g*, 30 min) and washed with 30 mM phosphate buffer, pH 7.6; a weighed portion of the biomass was diluted sixfold with the buffer; and the suspension was mixed with 2% agar gel precooled to 50°C (1 : 1). The obtained composition was applied to a Whatman GF/A glass fiber filter (Whatman, Great Britain), 3 × 3 mm, and air-dried for 15 min. This bioreceptor element was placed onto the surface of an oxygen electrode (Kronas, Russia) and fixed with a caprone net.

Biosensor detection of respiratory activity of the microorganisms. Cell respiration was registered by means of a flow injection biosensor system (Kronas, Russia) based on an oxygen electrode with immobilized bacterial cells of *P. putida* BS394(pBS268) applied to its surface. The sample volume was 0.05 ml; the flow rate was 0.6 ml/min. Total analysis time was 300 s; it was preceded by the phase of bringing the system into the measuring mode (100 s). The measurement was completed by a washing phase (100 s). Sodium–potassium phosphate buffer (pH 7.6) with salt concentrations of 30 mM was used in the work. The measured parameter (biosensor response) was the amplitude of current strength change. The results were processed using embedded software (Kronas, Russia).

Transformation of caprolactam oligomers by intact bacterial cells. The bacterium *P. putida* BS394(pBS268) was cultivated in a liquid Evans mineral medium with the addition of caprolactam oligomers (a dimer and a trimer of 6-aminohexanoic acid) as the sole carbon and energy sources (0.05–0.1%).

The process of oligomer transformation by the bacterial strain *P. putida* BS394(pBS268) was studied under long-term incubation of bacterial cells in the presence of the substrate. Cells of the degrader strain (logarithmic growth phase), 2.3×10^9 CFU/ml, were added to the phosphate buffer solution with a linear oligomer (2 mM). The mixture was incubated at 24°C for 24–48 h under continuous stirring. Samples taken from the reaction flask were centrifuged (10000 rpm, 12 min); the supernatant was separated, frozen, and stored at –20°C. The oligomers were incubated under the same conditions in the presence of cells of the strain *P. putida* BS394, which does not bear the plasmid pBS268.

Mass spectrometric analysis of the culture liquid. Measurements were performed in an LCQ Advantage MAX mass-spectrometer (Thermo Finnigan), with a single-channel syringe pump used for direct sample infusion into the ionization region. Mass spectrometry was performed under conditions of air pressure chemical ionization (APCI). The ion source working conditions were as follows: sheath gas flow rate 65 ml min^{-1} and capillary temperature 170°C. The normalized collision energy was 20–40%. The mass spectrometry data were collected and processed by the Xcalibur software package. Both positive and negative ions were detected, but the results were analyzed by negative ion spectra for the dimer and by positive ion spectra for the trimer (as more sensitive). MC/MC ions were obtained with the use of deprotonated $[M - H]^-$ molecules and protonated $[M + H]^+$ molecules as precursor ions for the dimer and trimer, respectively.

Determination of transaminase activity in bacteria. The activity of 6-aminohexanoate transaminase (EC 2.6.1) was assayed spectrophotometrically by the intensity of staining of the products of interaction between semialdehydes and 3-methyl-2-benzothiazolinone-2-hydrozone (MBTH) in the presence of iron(III) chloride at 580 nm 10 min after reaction initiation [14]. The measurements were performed with an SF103 spectrophotometer (Akvilon, Russia) in the kinetic mode. The enzyme quantity catalyzing the formation of 1 nmol of the MBTH and semialdehyde condensation product during 10 min at 25°C was accepted as a unit of activity. Molar absorption coefficient (ε) was taken as 50000 l mol^{-1} cm^{-1}. Protein was assayed according to Bradford [15].

Reagents. All reagents were graded as chemically pure or analytically pure (Sigma (United States), Merck (Germany), Amresco (United States), and Fluka (Switzerland)). The linear dimer and trimer of caprolactam were synthesized according to [16].

RESULTS AND DISCUSSION

P. putida BS394(pBS268) was chosen as the most active strain from the collection of caprolactam-degrading strains of the Laboratory of Plasmid Biology (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences). The strain was obtained by conjugative transfer of the caprolac-

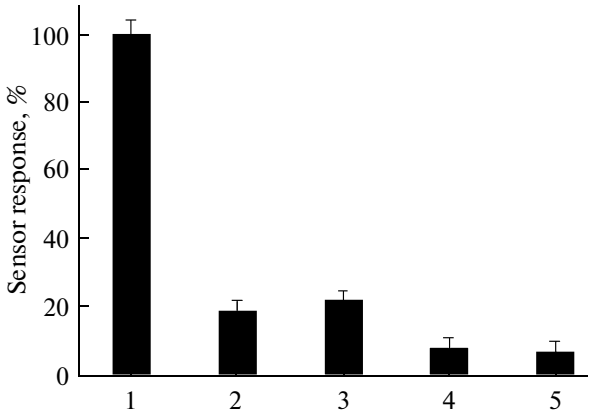

Fig. 1. Substrate specificity of *Pseudomonas putida* BS394(pBS268). The sensor response to respiration in the presence of 1 mM caprolactam was taken as 100%. Substrates (1 mM): caprolactam (1), dimer of 6-aminohexanoic acid (2), trimer of 6-aminohexanoic acid (3), aminocaproate (4), and adipate (5).

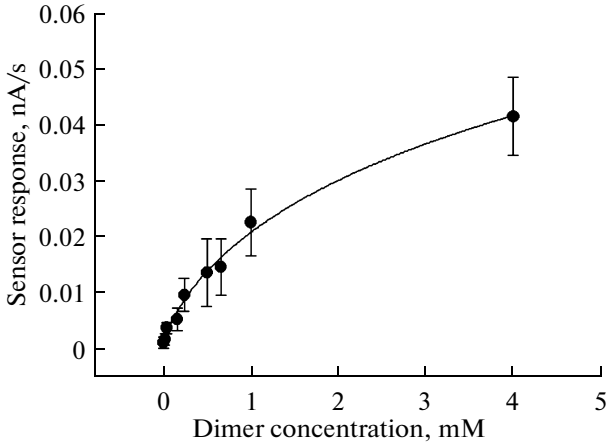

Fig. 2. Calibration dependence of the responses of the biosensor based on the strain *P putida* BS394(pBS268) on the concentration of the dimer of 6-aminohexanoic acid.

tam biodegradation plasmid pBS268 from the native degrader strain *P. putida* BS836 isolated from the territory of the Azot chemical enterprise (Severodonetsk, Russia) by the method of Dunn and Gunsalus [17]. The plasmid pBS268 (100 kb) belongs to the incompatibility group P-9 and determines the inducible synthesis of the enzymes responsible for caprolactam cleavage, 6-aminohexanoic acid transamination, and oxidation of adipic semialdehyde to adipate [18].

Assessment of the possibility of biodegradation of linear caprolactam oligomers by caprolactam-degrading bacteria. No growth of bacterial cells on the 6-aminohexanoic acid dimer or trimer as the sole carbon sources was observed; therefore, the strain *P. putida* BS394(pBS268) is unable to biodegrade linear oligomers. Caprolactam-degrading bacteria are possibly able to grow on oligomers under other cultivation conditions. It should be noted that [8] has demonstrated the possibility of realization of this feature by *Pseudomonas aeruginosa* PAO1 during long-term incubation on a minimal medium containing initially aminohexanoate and then the linear dimer as the sole carbon source.

Respiratory activity of the bacteria. The lack of ability to grow on caprolactam oligomers as an indication of their biodegradation by bacterial cells does not exclude the possibility of transformation, i.e., partial destruction, of these substrates, which essentially changes the structure of an organic substance but does not result in its complete utilization.

The biosensor method based on the measurement of respiratory activity of immobilized bacteria in the presence of the substrates was used to study the ability of bacteria to oxidize linear caprolactam oligomers. The principle of operation of an oxygen electrode-based microbial sensor is as follows: when caprolactam is oxidized by the microorganisms immobilized on the oxygen electrode surface, their respiratory activity increases and the decrease in oxygen concentration in the near-electrode space is registered by the electrode [19, 20]. The application of such biosensor systems has been proposed for rapid characterization of the physiological–biochemical behavior of microorganisms [21, 22], including comparative characterization of ε-caprolactam degrader strains with different CAP plasmid/bacterial host combinations [23].

Respiratory activity of the degrader strain *P. putida* BS394(pBS268) increased in the presence of not only caprolactam and its intermediates, but also its linear dimer and trimer (Fig. 1), indicating its ability to transform linear caprolactam oligomers under aerobic conditions.

It should be noted that the changes in respiratory activity of the cells of *P. putida* BS394(pBS268) depended quantitatively on oligomer concentrations. Figure 2 shows the calibration dependence of biosensor responses on concentration of the 6-aminohexanoic acid dimer. The lower detection limit was 0.02 mM.

These findings may serve as a basis for development of a biosensor for detection of caprolactam oligomers in aqueous media.

The genes of catabolism of these compounds in the pseudomonades degrading caprolactam oligomers are known to be localized on plasmids [11, 12]. Respiratory activity of the plasmid-free strain *P. putida* BS394 was studied to find out the localization of the genes controlling oligomer transformation in the strain under study. The respiratory activity of this bacterium was not observed to increase in the presence of the linear dimer, trimer, caprolactam, and its intermediates.

Biotransformation of linear caprolactam oligomers. Possible transformation pathways of caprolactam oligomers were ascertained by long-term (up to 48 h)

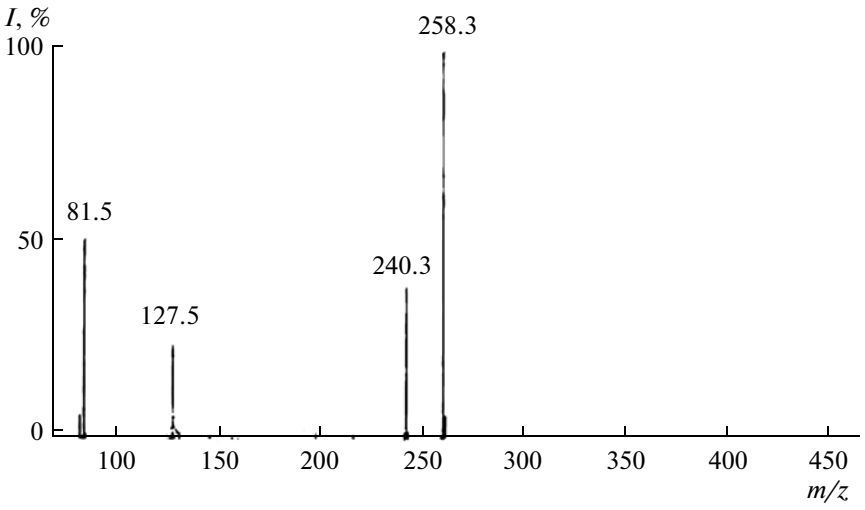

Fig. 3. MC/MC analysis of the peak $[M-H]^- = 258$ corresponding to the product of biotransformation of the dimer of 6-aminohexanoic acid (in negative ions, the normalized collision energy was 30%).

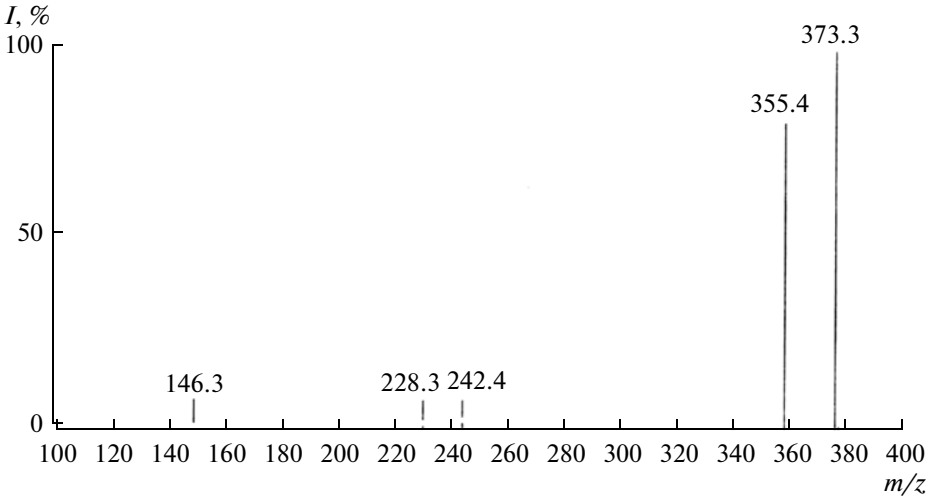

Fig. 4. MC/MC analysis of the peak $[M + H]^+ = 373$ corresponding to the product of biotransformation of the trimer of 6-aminohexanoic acid (in positive ions, the normalized collision energy was 21%).

incubation of *P. putida* BS394(pBS268), the caprolactam-degrading strain, in the presence of the linear dimer and trimer of 6-aminohexanoic acid with subsequent mass spectrometry for identification of the substances contained in the reaction mixture. After the incubation, the mass spectra of the culture liquid exhibited peaks corresponding to the molecular masses (Mr) of 259 and 372, which were absent in the mass spectra of the culture liquid at the beginning of incubation of bacteria with the linear oligomers. The molecular masses of these peaks coincided with the molecular masses of the products of oxidative transamination of the linear dimer and trimer of 6-aminohexanoic acid. Consequently, transformation of caprolactam oligomers results in formation of dicarboxylic acids, which are accumulated extracellularly in the culture liquid. The transformation products were identified by the results of MC/MC analysis of the peaks with Mr = 259 (Fig. 3) and Mr = 372 (Fig. 4).

Figures 5 and 6 present the schemes of fragmentation for neutral molecules of linear di- and trimers of 6-aminohexanoic acid.

It should be noted that the mass spectra of the reaction mixture exhibited no changes during the incubation of the plasmid-free strain *P. putida* BS394 with linear oligomers of 6-aminohexanoic acid. Hence, the transformation of linear oligomers in the studied caprolactam-degrading strain is under genetic control of the CAP plasmid pBS268.

These findings suggest that bacterial cells bearing the CAP plasmid are able to transform linear oligo-

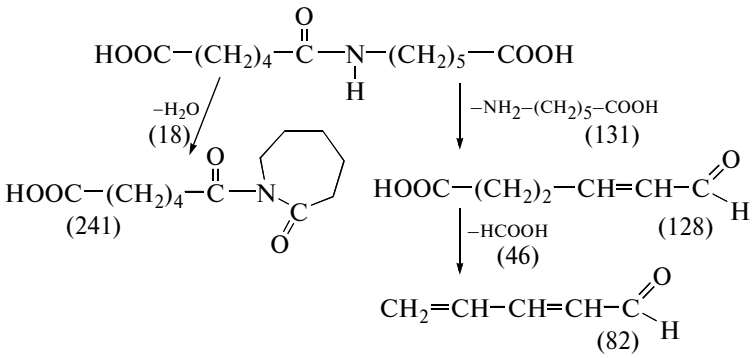

Fig. 5. Schemes of fragmentation of the product of biotransformation of the linear dimer of 6-aminohexanoic acid during the MC/MC analysis of molecular ions (molecular masses are given in parentheses).

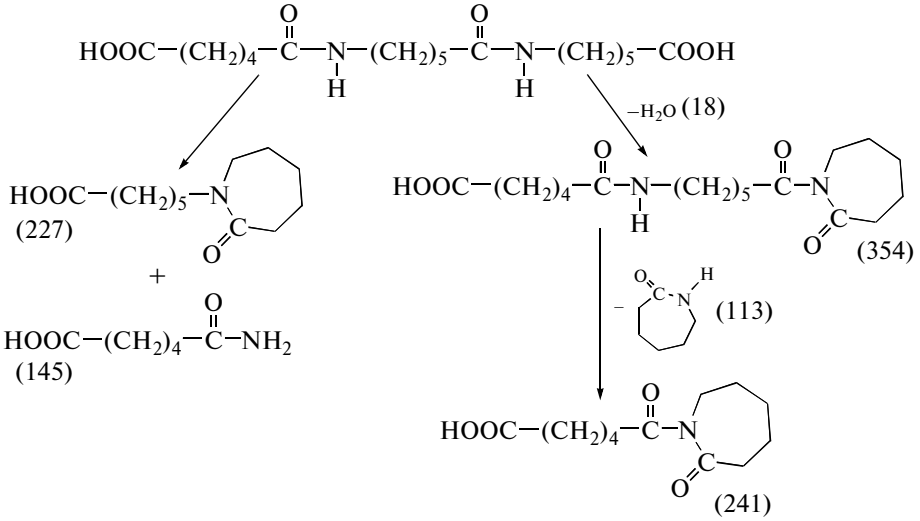

Fig. 6. Schemes of fragmentation of the product of biotransformation of the linear trimer of 6-aminohexanoic acid during the MC/MC analysis of molecular ions.

mers due to broad substrate specificities of two enzymes of caprolactam biodegradation: transaminase (EC 2.6.1-) and dehydrogenase (EC 1.2.1.63). This suggestion was verified by determining transaminase activity in the lysates of *P. putida* BS394(pBS268) cells in the presence of 6-aminohexanoate and the dimer of 6-aminohexanoic acid. It was shown that the specific transaminase activity was about two times lower toward the aminohexanoate dimer (0.48 nmol min^{-1} mg^{-1}) than toward the monomer, 6-aminohexanoate (0.91 nmol min^{-1} mg^{-1}). The calculated values of specific transaminase activities in the presence of both 6-aminohexanoate and its dimer suggest participation of the enzymes of caprolactam catabolism in the reactions of transformation of low-molecular oligomers of 6-aminohexanoic acid.

It is important to note that the biochemical reactions of microbial transformation of the linear oligomers of 6-aminohexanoic acid to the respective deriv-

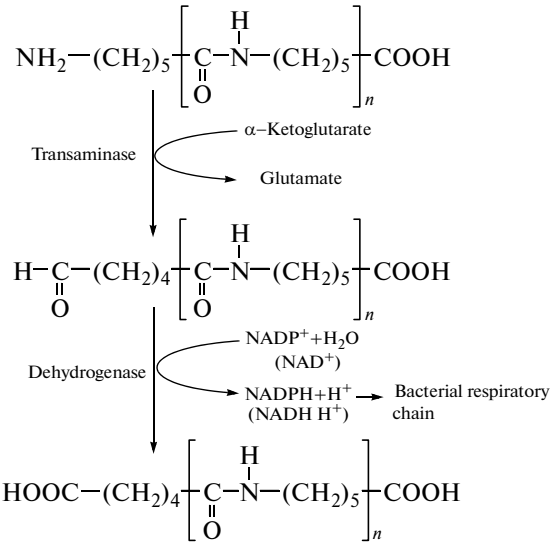

Fig. 7. The tentative scheme of transformation of the linear caprolactam oligomers by the bacterium *P. putida* BS394(pBS268).

atives with the terminal carboxyl groups have not been described previously.

Thus, the scheme of transformation of linear caprolactam oligomers by degrading bacteria *P. putida* is proposed for the first time as a result of the above studies (Fig. 7).

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