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Reshetilov, A N; Iliasov, P V; Fesay, A P; Ivashchenko, G V; Taranova, L A; Winther-Nielsen, M; Emnéus, Jenny

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Evaluation of Substrate Specificity of Biosensor Models Based on Strains Degrading Polycyclic Aromatic Compounds

A. N. Reshetilov[1], P. V. Iliasov[1], A. P. Fesay[2], G. V. Ivashchenko[2], L. A. Taranova[2], M. Winther-Nielsen[3], and J. Emneus[4]

[1]Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia
e-mail: anatol@ibpm.pushchino.ru
[2]Ovcharenko Institute of Biocolloid Chemistry, National Academy of Sciences of Ukraine, pr. Vernadskogo 42, Kiev, 252680 Ukraine
[3]DHI Water and Environment, 2970 Hoersholm, Denmark
[4]Lund University, Lund, Sweden

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Abstract—Models of microbial biosensors based on 11 strains of degraders of surface-active substances (SASs) and polycyclic aromatic hydrocarbons (PAHs) were studied. Substrate specificity, sensitivity, and stability of biosensor models were comparatively evaluated.

Polycyclic aromatic hydrocarbons (PAHs) constitute one of the most dangerous classes of pollutants. A number of derivatives of these compounds belong to the group of supertoxicants, i.e., compounds capable of exerting a toxic effect on a broad range of organisms even in concentrations 10^{-12}–10^{-9} M. In addition, many of these compounds have a cumulative effect, suppress immune and endocrine systems, have carcinogenic activity, etc. Combined environmental pollution with PAHs and surface-active substances (SASs) is of particular danger, because surfactants are able to solubilize substrates of many types. Besides, SASs constitute a large group of xenobiotics commonly found in industrial and household waste. Although surfactants are less dangerous than PAHs and surfactants in low concentrations readily degrade in the environment, their content in ecosystems of some regions is substantially above the normal level, and this fact itself is an environmental hazard.

Surfactants aggravate toxicity of polycyclic compounds by solubilizing them. In some cases, this process facilitates and accelerates their biodegradation by increasing the concentration of accessible xenobiotics in the aqueous phase [1]. The effect of acceleration of biodegradation of PAHs in the presence of SASs was reported for some degrader strains [2, 3] and successfully used in bioremediation processes, nonionogenic SASs (NSASs) being the most effective because of low toxicity and high rate of biodegradation [4, 5]. Thus, strains capable of mediating accelerated degradation of PAHs in the presence of SASs (nonionic detergents, in particular) are of significant interest for environmental biotechnology.

To provide an effective solution of the problem of environmental pollution, it is necessary to develop both methods of purification of contaminated ecosystems and rapid and reliable methods of pollutant detection. Although conventional methods of optical and chromatographic assay routinely used in laboratory settings provide high sensitivity and selectivity, they require sophisticated equipment and skilled personnel. On the other hand, biosensors, a branch of analytical biotechnology extensively developed during the last two decades, provide an opportunity to develop inexpensive and easy to use analytical devices. In addition to the relatively low cost and ease of use, biosensors are characterized by high sensitivity, reliability, and efficiency of chemical compound detection. Microbial biosensors, based on the cells of a specific microorganism, contain plasmids of biodegradation of the corresponding compound, and are regarded as promising tools for nature conservation applications.

A large number of laboratory models of microbial biosensors for nature conservation purposes have been described in the literature. These biosensors are able to detect various pollutants [6–9] and evaluate integral parameters of the environment, including the rate of biological oxygen demand (BOD) and total toxicity [10, 11]. The sensor model described in [8] was successfully used for detecting linear alkylsulfonates in water of the Ayaze River in Japan. It was shown in our earlier works [9, 12–14] that some bacterial strains which degrade anionic SASs (ASASs) were able to oxidize these substrates, making it possible to use an oxygen electrode as a biosensor transducer for monitoring SASs.

Overall, it seems more promising to use microbial strains with a broad spectrum of metabolic capacity in the biotechnology of water purification. Broad substrate specificity is thought to be the key factor of prac-

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tical applicability of microorganisms to biosensor analysis [15], because this property hampers the interference of other substances to be eliminated during analysis of actual samples [16] and accuracy of analysis to be increased. Therefore, a necessary stage in the biosensor system research is to study the substrate specificity of the systems with respect to major classes of organic xenobiotics capable of inducing analyzer signals and bringing about false results regarding actual samples.

The goal of this work was to evaluate parameters of biosensors that include receptor elements containing bacterial strains capable of mediating degradation of both PAHs and SASs, and to compare characteristics of these biosensors with characteristics of biosensors based on bacterial strains capable of degrading ASASs and NSASs, but lacking a pronounced ability to mediate degradation of PAHs. Additional goals of this work were to study the stability and degrader capacity of 11 strains with respect to SASs and polycyclic aromatic compounds, to evaluate substrate specificity of developed biosensor models, and to compare the obtained parameters with results described earlier for biosensor models based on bacterial degrader strains of ASASs and NSASs.

MATERIALS AND METHODS

Cultivation of microorganisms. The PAH-degrading strains used in this work were isolated in the Institute of Biocolloid Chemistry, National Academy of Sciences of Ukraine, within the framework of the project INCO-BIOFEED: *Pseudomonas putida* TI-Gj, *P. putida* TI-Ag, *Pseudomonas* sp. TF, *P. alkaligenes* TI-pyr, and degrader strains of SASs: *P. rathonis* T (degrader of volgonate), *Comamonas testosteroni* TI (degrader of nonylphenolethoxylate), *Pseudomonas* sp. TD (degrader of alkylbenzene sulfonate), *Pseudomonas* sp. 2T/1 (degrader of sodium dodecyl sulfate, SDS), *P. aeruginosa* 1C (degrader of alkylsulfates and SDS), *P. putida* K (degrader of methaupon, and *Achromobacter eurydice* TK (degrader of sodium monoalkylsulfosuccinate).

Cell cultures were grown on a rotary shaker (140 rpm) at 28°C in Erlenmeyer flasks containing synthetic liquid medium M9 with corresponding xenobiotic as the sole source of carbon and energy. Bacterial biomass was collected by centrifugation (5000 *g*, 20 min), washed three times with 30 mM potassium-phosphate buffer solution (pH 7.8), and used for preparing bioreceptor elements.

Substrates. The following compounds were used as substrates:

1. SASs (content of main substance, %; MS, %).

NSASs: nonylphenolethoxylate (NPE, 99); tween-60 (sorbitane monostearate, 90); tween-80 (sorbitane monooleate, 98); triton X-100 (*n*-(tret-octyl)phenol, 98); dodecyl ester of polyethyleneglycol (PEG), $n = 10$ and $n = 14$, 90; cetyl ester of PEG $n = 6$, 90; diethanolamide, 90; slovagen, 70; and sulfoethoxylate, 90.

ASASs: sodium dodecyl sulfate, SDS), 100; volgonate (alkylsulfonate, 60); methaupon (alkylmethyltaurine, 37); alkylnaphthalenesulfonate (ANS, 50), sodium monoalkylsulfosuccinate, 39; preparations of alkylbenzene sulfonate (ABS): ABS with linear, 90; and ABS with branched hydrocarbon chain, 80; decylbenzene sulfonate, 100; and chlorine sulfonol, 40.

Cationic SASs (CSASs): alkoxymethyldiethylammmonium methylsulfate (alkamone, 100); bis-quaternary ammonium ethonium, 100; cetyl pyridinium chloride, 100; tetradecylmethylammonium bromide, 100; alkyldimethylbenzene ammonium chloride (giamine, 90).

Ampholytic SASs (AmSASs): alkylaminobispropionate, 85; amidobetaine, 50; cyclimide, 60; and sulfobetaine SB14, 100.

2. Aromatic and polycyclic compounds: phenanthrene; anthracene; naphthalene; fluorene; pyrene; phenol; salicylate; benzoate, and catechol.

3. Carbohydrates: glycerol, glucose, xylose, and sucrose.

4. Alcohols: methanol, ethanol, propanol, and butanol.

5. Fatty acids (linolenic, myristic).

Biosensor assay. A receptor element was formed by incorporating bacterial cells in 2% agar gel using the method described in [9, 10]. The resulting receptor element (0.3- to 0.5-mm-thick membrane) was placed onto the measuring surface of a Clark oxygen electrode and attached with a capron mesh.

An Ingold 5313/010 amperometric system (Switzerland) was used as a biosensor transducer. Measurements were performed under continuous stirring in a 5-ml cuvette, at 20°C, and conditions of complete saturation with oxygen. The monitored parameter (sensor signal) was the maximal rate of output electric current change (nA/s) induced by substrate addition.

RESULTS AND DISCUSSION

Characteristics of strain P. putida T1-Gj. Stability and degrading capacity of the strain *P. putida* T1-Gj with respect to SASs and PAHs was evaluated in preliminary experiments. The stability of the strain was tested by the ability of the microbial cells to grow on standard culture media in the presence of degradable substrate, whereas the maximum concentration of substrate consumed by microbial culture was used as a measure of its degrading capacity. The results of these experiments are given in Table 1. Similar parameters of microbial strains which degrade SASs are also given in Table 1 for comparison.

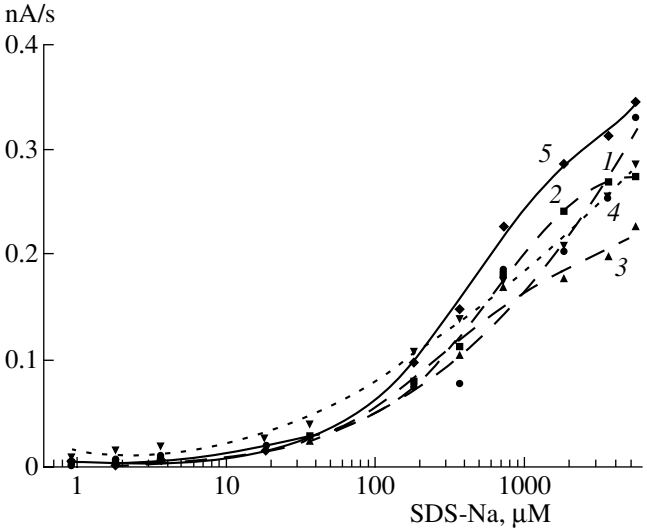
The strain *P. putida* T1-Gj was able to grow in the presence of any substrate tested at this stage of the study. Therefore, this strain was resistant to all types of

Table 1. Stability and degrading capacity of microbial strains with respect to SASs and PAHs

Substrate	Strain							
	P. putida TI-Gj		*Comamonas testosteroni* TI		*P. rathonis* T		*Pseudomonas* sp. TD	
	stability	degradation, mg/l	stability	degradation, mg/l	stability	degradation, mg/l	stability	degradation, mg/l
Volgonate	+	330	+	–	+	460	+	400
SDS	+	100	+	–	+	500	+	700
ABS	+	–	–	–	+	50	–	–
Ethonium	+	50	+	–	–	–	–	–
Amidobetaine	+	100	+	30	–	–	+	140
Cyclimide	N	N	N	N	–	–	+	440
NPE	+	–	+	300	+	400	–	–
Naphthalene	+	300	+	–	+	N	+	N
Pyrene	+	10	+	–	+	N	+	N
Fluorene	+	–	+	–	–	N	+	N
Phenanthrene	+	1.12	+	–	+	N	+	N
Anthracene	+	0.07	+	–	+	N	+	N

Notes: sign + denotes the ability to grow on standard media in the presence of substrate; sign – denotes the absence of growth; N denotes that the ability to grow was not tested.

SASs and polyaromatic compounds. This strain was also characterized by the ability to degrade the majority of SASs (except ABS and NPE) and PAH species tested. SASs were degraded in concentrations of about 50–300 mg/l, whereas concentrations of PAHs subjected to degradation ranged from 300 mg/l (naphthalene) to ~0.1–10 mg/l (tri- and tetracyclic compounds). According to these characteristics, the strains tested in this work are superior to the strains used earlier to degrade SASs. The previously used strains were not characterized by such a broad spectrum of degradable substrates but they were resistant to PAHs.



Dependence of signal amplitude (nA/s) on substrate concentration (μM) in biosensors based on bacterial strains degrading ASASs: (*1*) *P. rathonis* T; (*2*) *Pseudomonas* sp. 2T/1; (*3*) *P. aeruginosa* 1C; (*4*) *P. putida* K; (*5*) *A. euridice* TK.

Sensitivity and specificity of degraders of SASs and PAHs. During further stages of this work, cells of PAH-degrading bacterial strains were used to prepare receptor elements of a biosensor, which provided an opportunity to study the specificity of the strains in more detail with respect to SASs. Sensitivity and specificity of SAS degraders were tested in parallel experiments. Substrate specificity of these strains was tested using substrates belonging to different classes of SASs. The concentration of the substrates in these experiments was 100 and 1 mg/l. It was expected that compounds capable of exerting a possible toxic effect on bacteria in high concentrations could be available as substrates at low concentrations in medium. The volgonate-induced signals were taken as 100%. The results of the tests are given in Tables 2 and 3.

Biosensors based on SAS-degrading bacterial strains are characterized by high sensitivity to these compounds. Calibration curve for SDS is shown in figure. The lower threshold of SDS detection for biosensors based on *P. rathonis* T, *P. putida* K, and *A. eurydice* TK was 0.25 mg/l (0.86 μM), whereas for biosensors based on *Pseudomonas* sp. 2T/1 and *P. aeruginosa* 1C it was 0.5 mg/l (1.73 μM). The dependence of the electric current changes (nA/s) and concentration of SDS was linear within the range from 0.25 to 200 mg/l. It follows from the figure that at a concentration of 734 μM, the amplitudes of the SDS-induced responses

Table 2. Relative specificity of biosensors based on microbial strains degrading SASs (arithmetic mean averaged over three experiments)

Substrate, 100 mg/l	*P. rathonis* T	*Pseudomonas* sp. 2T/1	*P. aeruginosa* 1S	*P. putida* K	*A. eurydice* TR
Volgonate	100	100	100	100	100
SDS	117	121	81	122	119
Sodium monoalkylsulfosuccinate	150	223	81	194	156
Alkylnaphthalenesulfonate	42	5	0	18	32
Decylbenzene sulfonate	44	9	8	18	14
Methaupon	24	2	6	35	8
ABS	15	5	0	36	6
ABS with linear chain	20	3	1	17	5
ABS with branched chain	0	0	0	0	0
Dodecyl ester of PEG, $n = 10$	52	20	27	30	33
Dodecyl ester of PEG, $n = 14$	28	16	28	28	35
Cetyl ester of PEG, $n = 6$	7	0	0	12	22
NPE	28	10	41	8	16
Slovagen	14	0	9	0	6
Triton X-100	3	0	16	0	0
Tween-60	33	21	31	38	37
Tween-80	28	51	35	21	41
Sulfobetaine SB14	140	36	47	56	115
Alkamone	12	0	17	0	0
Tetradecylmethylammonium bromide	24	13	52	0	0
Cetyl pyridinium chloride	0	0	0	0	0
Myristic acid	0	0	0	0	0
Linolenic acid	36	85	60	24	60
Phenol	0	0	0	0	0
Salicylate	0	3	0	15	4
Benzoate	0	0	0	0	0
Catechol	0	0	33	0	0
Glucose	7	28	11	25	8
Xylose	0	0	0	0	0
Sucrose	0	0	0	0	0
Glycerol	0	0	0	0	0
Ethanol	68	12	19	78	198
Propanol	53	7	21	30	34
Butanol	21	18	18	30	64
Methanol	0	0	0	0	0

for all strains were close to each other. It should be noted that the broad range of detectable concentrations of SDS (0.25–1500 mg/l) makes it feasible to use these biosensors for detecting ASASs in industrial wastes, household wastewater, and natural waters.

Regarding SAS-degrading bacterial strains (Tables 2 and 3), it should be emphasized that they were characterized by similar specificity (except *C. testosteroni* TI) and high sensitivity to the majority of CSASs, ASASs, and NSASs. The confidence level of the SAS-induced responses of the strains was assessed using the Student's test. The mean value of the confidence level was 0.61, the strains *P. rathonis* T, *Pseudomonas* sp. 2T/1, *A. eurydice* TK, and *P. putida* K being characterized by the largest similarity (confidence level for data on specificity to SASs exceeded 0.92). Signal stability in the case of CSASs differed from that for the other sub-

Table 3. Relative specificity of biosensors based on microbial strains degrading PAHs and SASs (arithmetic mean of three experiments)

Substrate	*P. putida* TI-Gj		*P. putida* TI-Ag		*Pseudomonas* sp. TF		*P. alcaligenes* TI-pyr		*Pseudomonas* sp. TD		*C. testosteroni* TI	
	I	II	I	II	I	II	I	II	I	II	I	II
Volgonate	100	100	100	100	31	27	100	100	20	28	88	48
SDS	115	129	77	86	23	29	85	106	27	37	105	55
Methaupon	55	76	0	59	9	0	0	54	29	33	57	52
Alkylnaphthalenesulfonate	38	120	31	35	0	0	85	94	0	0	5	0
Sodium monoalkylsulfosuccinate	68	107	110	45	10	12	32	56	45	33	127	23
ABS	8	0	24	0	10	10	75	54	100	100	94	10
ABS with linear chain	7	0	22	0	7	0	50	0	78	56	35	13
ABS with branched chain	0	0	0	0	0	0	0	0	0	0	0	18
NPE	18	0	0	0	100	100	36	0	12	0	100	100
Tween-80	28	22	0	52	15	0	0	0	0	0	90	40
Triton X-100	7	0	0	0	24	0	31	0	0	0	95	98
Dodecyl ester of PEG, $n = 10$	24	80	12	26	15	0	28	0	0	0	90	133
Dodecyl ester of PEG, $n = 14$	21	62	20	35	0	0	45	33	0	0	81	23
Cetyl ester of PEG, $n = 6$	0	0	22	29	0	0	0	0	0	0	52	25
Diethanolamide	0	0	0	0	7	11	0	33	0	0	83	28
Sulfoethoxylate	0	0	0	0	8	0	0	0	0	0	45	0
Ethonium	20	82	0	0	5	13	31	39	0	0	47	75
Cetyl pyridinium chloride	0	0	0	0	0	0	0	0	0	0	35	0
Tetradecylmethylammonium bromide	0	0	0	0	0	0	0	0	0	0	0	40
Gyamine	0	0	0	0	0	0	0	0	0	0	0	30
Alkamone	0	0	0	0	0	0	0	0	0	0	0	99
Alkylaminobispropionate	0	0	0	21	0	0	0	0	0	0	13	0
Amidobetaine	85	89	33	52	0	0	61	50	21	36	40	36
Glucose	16	91	67	93	87	86	138	111	50	41	0	0
Ethanol	25	78	53	50	50	43	30	52	60	44	84	92
Propanol	12	0	30	0	10	0	20	0	0	0	54	85

Note: Substrate concentration (mg/l): I, 100.0; II, 1.0.

strates: both ASAS- and NSAS-induced signals were maintained at a constant level throughout the whole period of receptor element functioning, whereas the CSAS-induced signal was detected only after the first exposure to substrate, and then the activity of the receptor element was lost. Perhaps this was caused by pronounced antimicrobial effect of CSASs [17, 18]. Of all compounds listed above (besides SASs), these strains responded only to naphthalene, ethanol, and propanol.

Regarding the selectivity of bacterial strains of degraders, strains *P. rathonis* T and *Pseudomonas* sp. 2T/1 should be particularly noted, because they had the narrowest substrate specificity and highest sensitivity to SDS and some SAS species of other classes. It was found that a biosensor based on *P. rathonis* T and *Pseudomonas* sp. 2T/1 responded specifically to monoalkylsulfosuccinate (about 100% of SDS-induced signal for the two strains), alkylsulfonate (about 50% of SDS-induced signal for the two strains), dodecyl ester of polyethyleneglycol $n = 10$ (41% for *P. rathonis* T), tween-80 (about 20% for *Pseudomonas* sp. 2T/1), alkamone (100% for *P. rathonis* T and 15% for *Pseudomonas* sp. 2T/1), naphthalene (70% for *P. rathonis* T), and certain fatty acids and carbohydrates. Responses of these strains to alcohols did not exceed 60% of the SDS-induced response: for ethanol, 50% for propanol, and 18% for butanol in case of the use of *P. rathonis* T and 10% for ethanol, 5% for propanol, and 15% for butanol in case of the use of *Pseudomonas* sp. 2T/1. In addition to the high value of the response induced by ASASs, the biosensor based on *C. testosteroni* TI is also sensitive to NSASs and some CSASs, which makes it promising for integral evaluation of the SAS concentration in a sample. Thus, considering the prospects of practical use for the bacterial cultures studied in this work in biosensors designed to detect SASs, it is

safe to conclude that the selectivity and calibration characteristics of bacterial strains provide satisfactory parameters of biosensor assay. Therefore, these strains can be used in biosensors for detecting SASs in actual samples.

All PAH-degrading strains studied in this work were also characterized by high sensitivity to ASASs, particularly at low concentrations of these compounds (Table 3). In addition, strain *Pseudomonas* sp. TF was characterized by a high sensitivity to NSASs, low concentrations of these compounds being unable to induce biosensor response. Perhaps this strain was an active degrader of NSASs, and it was resistant to the toxic effect of these compounds. The other bacterial cultures tested in this work were characterized by low sensitivity to NSASs. The strain *P. putida* TI-Gj was an exception to this rule. This strain generated large-amplitude signals in the presence of low concentrations of dodecyl esters of PEG, whereas the signals generated by the strain in the presence of high concentrations of dodecyl esters of PEG did not exceed 25% of the volgonate-induced response. None of the CSASs, except ethonium, induced a biosensor response. The ethonium-induced response was observed for all strains except *P. putida* TI-Ag, the signal generated by the strain *P. putida* TI-Gj in the presence of 1 mg/l ethonium being 82% of the volgonate-induced response. The AmSAS amidobetaine also induced a large response in biosensors based on all bacterial strains but *Pseudomonas* sp. TF. However, alkylamino-bis-propionate, which also belongs to the same group of compounds, induced virtually no response in these strains.

Levels of substrate specificity of strains *P. putida* TI-Gj, *P. putida* TI-Ag, and *P. alcaligenes* TI-pyr differed only insignificantly from one another, which represented the characteristic organization of each of their enzyme systems and, perhaps, genetic systems responsible for SAS metabolism. The mean confidence level of a comparison of the SAS-induced responses of the strains, assessed using the Student's test, was 0.54. It should also be noted that there was a considerable similarity in the level of substrate specificity of these strains with the substrate specificity of degraders of ASASs, *Pseudomonas* sp. 2T/1, *P. aeruginosa* 1C, and *A. eurydice* TK (according to the Student's test, mean confidence level for the difference between the SAS-induced responses of the strains was 0.75–0.80). This suggests that these strains can be used in biosensors for detecting ASASs. On the other hand, it should be taken into account that the broad spectrum of substrate specificity of the biosensor based on the strain *P. putida* TI-Gj makes it reasonable to use this biosensor for assaying the total content of SASs. It was noted above that the strain *Pseudomonas* sp. TF could be used as a component of a biosensor for detecting NSASs, NPE in particular. Its comparison with *C. testosteroni* TI, an NPE-degrading strain isolated earlier, revealed its higher selectivity with respect to the latter. In some cases, this makes the use of the strain more advantageous.

It is well known that low selectivity is the main disadvantage of microbial biosensors [18]. However, in ecological monitoring it is often more important to determine the whole pool of xenobiotics present in an aquatic ecosystem rather than to identify specific pollutants. The use of differential detection mode and image recognition approaches [19] (e.g., by assaying a sample with several electrodes in one cell) makes it possible not only to increase the selectivity of measurements but also to identify different classes of SASs. For example, the use of the three-electrode biosensor system based on *P. rathonis* T, *Pseudomonas* sp. 2T/1, and *P. aeruginosa* 1C reveals with sufficient accuracy whether or not a given sample contains dodecyl ester of polyethyleneglycol, $n = 14$ (by the presence of a response in the biosensor based on *P. rathonis* T *P. aeruginosa* 1C and the lack of a response in the biosensor based on *Pseudomonas* sp. 2T/1) or tetradecylmethylammonium bromide (by the presence of a response in the biosensor based on *P. aeruginosa* 1C and the lack of a response in the biosensor based on *P. rathonis* T and *Pseudomonas* sp. 2T/1).

Operating stability of biosensors. The operating stability of biosensors (functional duration of one receptor element) was assessed by repeated measurement of SDS (in concentration 200 mg/l) during a period of 3–5 days. During this period, the biosensor was kept in a buffer solution at room temperature under continuous stirring. The biosensor signal decrease during this period was insignificant. In case of *P. rathonis* T, *Pseudomonas* sp. 2T/1, and *P. aeruginosa* 1C this decrease did not exceed 5–10%, whereas in the case of *P. putida* K and *A. eurydice* TK, it did not exceed 20% within 24 h. The storage stability of strains was assessed during receptor element storage by measuring the responses of different receptor elements induced by SDS (concentration, 200 mg/l) within a period of 5–6 days. The highest activity during storage in agar was conserved in strains *P. rathonis* T, *Pseudomonas* sp. 2T/1, and *P. aeruginosa* 1C. The decrease in the signal activity in these strains was observed only after 5–6 days of storage in agar.

Because some strains considered in this work were PAH-degrading, the next necessary stage of the study was to evaluate the specificity of the biosensor models developed in this work with respect to mono-, di-, and polycyclic aromatic compounds. If high sensitivity and specificity with respect to PAHs were revealed in some strains, it would be promising to develop differential detection systems including biosensors of PAHs and different classes of SASs on the basis of these strains. Such a system could provide a basis for development of analyzers for environment monitoring in regions contaminated with PAHs.

Thus, all strains tested in this work are degraders of ASASs and resistant to the other classes of surface-active substances. Comparative analysis of biosensors based on these strains demonstrated significant similar-

ity of the parameters of biosensors designed to detect different organic substrates and a high sensitivity of these biosensors with respect to sodium dodecyl sulfate, volgonate, and some other species of ASASs. Most cultures studied in this work were inactive with respect to CSASs or NSASs and can be used either to detect ASASs in the presence of PAHs or as a component of the differential detection system containing biosensors sensitive to PAHs and various classes of SASs. In addition, biosensors based on strains *Pseudomonas* sp. TF and *C. testosteroni* TI were found to be able to detect the presence of NSASs and used for their selective assay. It seems promising to evaluate the selectivity of the biosensor models with respect to a broad spectrum of aromatic compounds.

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