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MONOAROMATIC COMPOUNDS BIODEGRADATION BY HYDROCARBON-OXIDIZING ACTINOBACTERIA

Monoaromatic substances belong to the most widespread and dangerous environmental pollutants. They are components of wastewater from oil refineries and the coal and chemical industries and are often found in soil, groundwater, and surface water. The use of actinobacteria for bioremediation of natural and industrial objects contaminated by these substances is an environmentally and economically advantageous alternative to physicochemical cleaning methods. Therefore, it is actual to search for new actinobacteria strains capable to assimilate of monoaromatic compounds for their further use in biotechnologies of purification of the environment polluted by these substances. ***Aim.*** *To determine the ability of hydrocarbon-oxidizing strains of actinobacteria of the Ukrainian Collection of Microorganisms to assimilate monoaromatic compounds — derivatives of benzene and phenol.* ***Methods****. The strain cultivation was carried out in a liquid mineral medium at an initial concentration of monoaromatic compounds of 500 mg/L as the only source of carbon and energy. The expression activity of the catA gene encoding catechol 1,2-dioxygenase, the key enzyme at the initial stage of the monoaromatic compounds biodegradation, was assessed under culture growth conditions with phenol and glucose. Relative expression of the catA gene was evaluated using RT-qPCR. Fatty acid methyl esters were obtained by hydrolysis of cells in a 5 % solution of acetyl chloride in methanol, followed by extraction with an ether-hexane mixture. Methyl esters were identified using an Agilent 6800N/5973 inert GC/MS system (Agilent Technologies, US). Fatty acid content was determined by AgilentChemStation software.* ***Results.*** *It was established that the studied strains of actinobacteria belonging to the species Dietzia maris, Gordonia rubripertincta, Rhodococcus aetherivorans, and Rhodococcus erythropolis differ in their ability to assimilate monocyclic aromatic compounds — benzene and phenol derivatives. Most strains grew better in media with benzene and its derivatives. All strains well grew on a mixture of ethylbenzene, ortho-, meta-, and para-xylene (EX), most of them grew with different intensities on a mixture of benzene, toluene, and ortho-xylene (BTX), and on monosubstrates — benzene, benzotriazole, and benzoate. Toluene was used by 50% of the studied strains, and only one of them (R. aetherivorans UCM Ac-602) grew on ortho-xylene. A smaller amount of strains grew in media with phenol derivatives. They did not assimilate ortho-cresol and hydroquinone but grew with different intensities on phenol, paranitrophenol, resorcinol, and catechol. Only a few strains grew in the presence of meta- and para-cresols. The widest*

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range of monoaromatic substances was used by R. aetherivorans UCM Ac-602. It was shown that in the process of phenol assimilation by strain R. aetherivorans UCM Ac-602, the level of transcriptional activity of the catA gene encoding the key enzyme of an ortho-pathway of aromatic ring cleavage, catechol 1,2-dioxygenase, increased almost 2-fold after 48 h compared to 24 h of strain growth on this substrate and 3-fold compared to glucose. The obtained data indicate the ability of R. aetherivorans UCM Ac-602 strains to assimilate monoaromatic substances through theortho-cleavage way. During growth on benzene, toluene, BTX and EX substances, the fatty acid pool of cells of this strain was dominated by hexadecanoic C16:0 (32.73-39.81 %), hexadecenoic C16:1 cis-9 (5.66-15.11 %), and octadecenoic C18:1 cis-9 (7.09-11.83 %) acids, as well as 10-methyloctadecanoic 10-Me-C18:0 (29.88-34.76 %) acid. The composition of fatty acids of cells grown on aromatic compounds, unlike those grown on glucose, contained 2.4-4.0 times less unsaturated C18:1 cis-9 acid and 2.8-3.2 times more methyl-branched acid 10Me-C18:0. ***Conclusions.*** *Assimilation of monoaromatic compounds — derivatives of benzene and phenol - is a strain-specific characteristic of the actinobacteria species. Using strain R. aetherivorans UCM Ac-602 as an example, it was shown that the assimilation of monoaromatic substances in studied actinobacteria occurs through the ortho-cleavage pathway. One of the adaptation mechanisms of strain R. aetherivorans UCM Ac-602 to the assimilation of these substances is a significant decrease in unsaturated fatty acids and an increase in methyl branched acids, which contributes to an increase in membrane rigidity and cell resistance to the toxic effect of these substrates. The data obtained indicate a high level of adaptation of actinobacteria to the assimilation of monoaromatic compounds and the prospects for their further study to be used in biotechnologies for environmental purification from these pollutants.*

Keywords: *actinobacteria, Rhodococcus, Dietzia, Gordonia, monoaromatic substances, fatty acids, catA gene, catechol 1,2-dioxygenase.*

Monoaromatic hydrocarbons, in particular, benzene, toluene, ethylbenzene, and xylenes (BTEX substances), as well as phenolic compounds, belong to the most environmentally hazardous group of organic pollutants by leakage volume and risks to human health. The toxic effect of these substances on humans is associated with their carcinogenicity and disruption of the endocrine and central nervous systems (Orazbayeva et al., 2016; Krivoruchko et al., 2023). Aromatic substances are found in almost all technogenic and natural objects. Most industrial wastes contain various organic mixtures, which makes it important to study the microbial degradation of composite substrates. The source of pollution of soil and surface and groundwater with aromatic substances is oil spills, wastes of the petrochemical and chemical industries, including the production of fuels, petroleum products, explosives, resins, pesticides, herbicides, paints, pharmaceuticals, dyes, vehicle exhaust emissions, and others (Bolden et al., 2015; Feng et al., 2021; Ali et al., 2023).

Biological methods based on the use of microorganisms capable of assimilating these toxicants as the only source of carbon and energy are widely used for the utilization and detoxification of aromatic substances. The advantage of biological methods for cleaning from such contaminants over chemical and physical ones is their relatively low cost, low energy consumption, and environmental safety (Panigrahy et al., 2022). Under aerobic conditions, aromatic compounds are able to be assimilated by representatives of different genera of bacteria, in particular, *Pseudomonas*, *Rhodococcus*, *Nocardia*, *Micrococcus*, *Bacillus*, *Arthrobacter*, *Acinetobacter*, and others (Di Canito et al., 2018; Panigrahy et al., 2022). Actinobacteria of the genus *Rhodococcus* are among the most promising microorganisms for use in bioremediation of aromatic substances contaminating environments. *Rhodococcus* can biodegrade a wide range of toxic organic pollutants, including petroleum hydrocarbons, polychlorinated biphenyls, pharmaceutical pollutants, pesticides, explosives, flame retardants, plasticizers, defoliants, dyes, and microplastics (Krivoruchko et al., 2023). They exhibit a high resistance to various classes of toxic substances, maintain high metabolic activity under unfavorable conditions, and are able to synthesize environmentally important metabolites, which leads to the widespread commercial use of these microorganisms for biodeg-

radation and biotransformation of persistent organic environmental pollutants. In addition, the genus *Rhodococcus* contains a small number of opportunistic species and pathogens, representing an advantage from the viewpoint of safety (Kim et al., 2018; Cappelletti et al., 2020; Nazari et al, 2022). For the metabolism of toxic aromatic pollutants, actinobacteria use a complex of adaptations, including mechanisms of resistance to chemical toxicants, cellular transport systems for these substances, and specific catalytic enzymes.

In aerobic degradation, aromatic compounds are often oxygenated and transferred to dihydroaromatic derivatives such as catechol, gentisate, protocatechuate, and homogentisate. Catechol, which is a key intermediate in the initial stages of biodegradation of various aromatic compounds, can be metabolized by various microorganisms through *ortho*-cleavage with the enzyme catechol 1,2-dioxygenase or *meta*-cleavage with the enzyme catechol 2,3-dioxygenase (Táncsics et al., 2008; Paisio et al., 2014; Krivoruchko et al., 2023). The *ortho*- and *meta*-pathways depend not only on the types of substrates but also on their concentration in the same microorganism. For instance, at a low concentration of benzoate (200—300 mg/l), *Pseudomonas putida* activates only the *ortho*-pathway whereas both *ortho*- and *meta*-degradation pathways are activated at higher concentrations (Panigrahy et al., 2022). The literature data indicate the presence in many species of rhodococci, in particular, *R. erythropolis*, *R. qingshengii*, *R. opacus*, *R. pyridinivorans*, *R. rhodochrous,* and *R. ruber* of the catechol 1,2-dioxygenase enzyme, encoded by the *catA* gene (Kim et al., 2002; Táncsics et al., 2008; Krivoruchko et al., 2023). Estimating the abundance or expression of this gene during the growth of strains in media with monoaromatic substances, it is possible to obtain a potential measure of activity and determine possible ways of their biodegradation.

An important role as a protective barrier in the adaptation of bacteria to the action of aromatic substances is played by the lipids of the cell's cytoplasmic membranes, which contain fatty acids as a main component. Accumulation of toxic substances in bacterial membranes can lead to increased cell membrane fluidity, which disrupts the phospholipid bilayer and interferes with membrane-bound proteins, ultimately causing cell death (De Carvalho, 2019). As a rule, all adaptive mechanisms activated by the cell in response to changes in environmental conditions are associated with maintaining the membrane fluidity at a constant level, which is achieved by changes in the spectrum of the membrane's fatty acids. It is known that the main components of membrane lipids are unsaturated fatty acids, and an increase in the degree of their saturation is one of the main reactions of bacteria to the presence of aromatic compounds in the environment (Garba et al., 2016; De Carvalho, 2019; Shipko & Duvanova, 2019).

The work aimed to determine the ability of hydrocarbon-oxidizing strains of actinobacteria from the Ukrainian Collection of Microorganisms (UCM) to assimilate monoaromatic substances — derivatives of benzene and toluene.

Materials and Methods. The objects of the study were collection strains of hydrocarbon-oxidizing actinobacteria — representatives of the species *R. erythropolis* UCM Ac-23, UCM Ac-51, UCM Ac-77, and *R. aetherivorans* UCM Ac-602, maintained in the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine (IMV NANU). In this work, we also used strains of *R. erythropolis* IMV B-7012 (UCM Ac-29), *R. erythropolis* IMV B-7277 (УКМ Ac-603), *D. maris* IMV B-7278 (UCM Ac-205) and *G. rubripertincta* IMB Ac-5005 (UCM Ac-125), maintained in the Depository of the IMV NAS of Ukraine and are part of the preparation «Ekolan-M», intended to cleaning the environment from oil pollution.

The following aromatic substances were used in the studies: benzene and its derivatives — toluene, *ortho*-xylene, a mixture in equal proportions of benzene, toluene, and *ortho*-xylene (BTX substances), a mixture in equal proportions of ethylbenzene, *ortho*-, *meta*- and *para*-xylene

(EX substances), sodium benzoate, and benzotriazole, as well as phenol and its derivatives — *ortho*-, *meta*- and *para*-cresol, *para*-nitrophenol, catechol, resorcinol, and hydroquinone. The ability of strains to assimilate monoaromatic compounds was determined in a liquid mineral medium (g/L): NH_4NO_3, 0.75; Na_2HPO_4, 0.73; KH_2PO_4, 0.35; $NaHCO_3$, 0.25; $MgSO_4 \times 7H_2O$ — 0.02, pH — 7.0 (Nogina et al., 2020). Monoaromatic compounds (0.5 g/L) were used as the sole source of carbon and energy. Cultivation was carried out in flasks with a volume of 750 mL on shakers (220 rpm) at a temperature of 28 °C. The inoculum was an aqueous suspension of 48 h bacterial cultures grown on an agar medium (g/L): peptone — 10.0, yeast extract — 5.0, glucose — 5.0, NaCl — 5.0, agar — 15 (https://www.dsmz.de/?id=441). Bacterial growth after 7 days of cultivation in a liquid medium was measured by optical density at 540 nm using a photocolorimeter KFK-2MP. Solutions without inoculated media were used as references.

Determination of possible ways of the benzene ring biodegradation during benzene, toluene, EX, and BTX assimilation by actinobacteria strains was carried out by the Rothera reaction, as previously described (Erdoğmuş et al., 2013). The expression of the *catA* gene, which encodes catechol 1,2-dioxygenase, the key enzyme of the *ortho*-pathway of benzene ring cleavage, was investigated on the example of *R. aetherivorans* strain UCM Ac-602 during its growth on phenol. Isolation of total RNA from cells of the strain was performed using Trizol Reagent (Invitrogen) and subsequent treatment with DNase I (Fermentas, Lithuania). The RNA concentration was determined using a DS-11 FX+ device (DeNovix, USA). cDNA was synthesized from 0.5—1 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific), which contained a mixture of random primers and M-MLV revertase. Quantitative amplification was performed in a 10 μL PCR mix containing 5 μL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™), 20 pmol of each primer (*catA*-F: 5'-CTACTACTCCCAGTTCGCC-3'and *catA*-R: 5'-GCGAAGTAC-AGCTGGGTGAT-3'), and 3 μL cDNA template. The nucleotide sequences of primers for amplification of the *catA* gene fragments were selected using the MEGA 5.0 (Tamura et al., 2011) and Primer3 programs based on the known sequences of this gene in representatives of the genus *Rhodococcus*, available in the GenBank and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. Real-time PCR amplification was performed on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) with the temperature regime recommended by the manufacturer. The specificity of the target product was determined by agarose gel electrophoresis and melting curve analysis. The relative level of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The expression of the 16S rRNA gene was taken as an endogenous control (Zelena et al., 2014).

Fatty acid methyl esters of cells were obtained by cell hydrolysis in a 5% acetyl chloride solution in methanol for 4 h at 100 °C. Samples were kept at 80 °C for 2 h. Methyl esters were extracted three times with heptane and analyzed by gas chromatography-mass spectrometry on an Agilent 6890N/5973 inert chromato-mass spectrometric system (Agilent Technologies, USA) under the conditions: capillary column HP-5ms 30m×0.25mm×0.25mkm (Agilent technologies, USA); temperature range 150—250 °C; temperature gradient 4 °C/min; carrier gas helium; flow rate through the column 1.2 mL/min; the evaporator temperature 250 °C; the sample was injected in the mode with a flow split of 1:100. Identification of methyl esters was performed using the NIST02 mass spectrum library and a standard mixture of fatty acid methyl esters (47080-U, Supelco, USA) and expressed as a percentage of the total peak area.

All experiments were performed in triplicate. The obtained data were analyzed with Microsoft Office Excel 2010 software standard package.

Results. Examination of the ability to assimilate monoaromatic substances showed that all strains better utilized BTX substances, and a smaller amount of them grew in the presence of benzotriazole and EX substances (Table 1). In contrast, the strains (exept *R. aetherivorans* UCM Ac-602) did not use *ortho*-xylene monosubstrate. Only 4 strains showed moderate growth in the presence of toluene: *R. erythropolis* UCM Ac-29, UCM Ac-603, *R. aetherivorans* UCM Ac-602, and *G. rubripertincta* UCM Ac-125. Strains *R. aetherivorans* UCM Ac-602 and *G. rubripertincta* UCM Ac-125 moderately grew on sodium benzoate and *R. erythropolis* UCM Ac-603, and *D. maris* UCM Ac-205 did not grow in the medium with this substrate. It was found that *R. aetherivorans* UCM Ac-602 better assimilate BTX and EK substances. *R. erythropolis* UCM Ac-23 and *R. aetherivorans* UCM Ac-602 moderately utilized benzene; all other strains (except for *R. erythropolis* UCM Ac-77) grew weakly in the presence of this substrate.

It was found that tested strains grew weakly in the medium with phenol derivatives. All strains did not use *ortho*-cresol and hydroquinone. In the medium with *meta*-cresol, good growth was found in strain *R. erythropolis* UCM Ac-602, poor growth — in *R. erythropolis* UCM Ac-51 and UCM Ac-77, but the other strains did not use this substrate at all. Strains *R. aetherivorans* UCM Ac-602 and *G. rubripertincta* UCM Ac-125 were

Table 1. **Growth of hydrocarbon-oxidizing actinobacteria strains when using monoaromatic compounds as the only source of carbon and energy**

Substrate	Strains							
	R. erythropolis					*R. aetherivorans*	*D. maris*	*G. rubripertincta*
	UCM Ac-23	UCM Ac-29	UCM Ac-51	UCM Ac-77	UCM Ac-603	UCM Ac-602	UCM Ac-205	UCM Ac-125
Benzene and its derivatives								
Benzene	++	+	+	—	+	++	+	+
Toluene	—	++	—	—	++	++	—	++
Ortho-xylene	—	—	—	—	—	+	—	—
BTX substances	—	++	+	+	—	+++	—	+
EX substances	+++	++	++	++	+++	+++	+	++
Sodium benzoate	+	+	+	+	—	++	—	++
Benzotriazole	++	++	+++	+++	—	—	+	+++
Phenol and its derivatives								
Phenol	+	—	+++	—	+	+++	—	+++
Ortho-cresol	—	—	—	—	—	—	—	—
Para-cresol	—	—	—	—	—	++	—	++
Meta-cresol	—	—	+	+	—	+++	—	—
Para-nitrophenol	+	—	++	—	—	+	+	++
Catechol	—	+	+	—	+	+	—	+
Resorcinol	—	++	—	—	+++	++	—	+
Hydroquinone	—	—	—	—	—	—	—	—

«—» no growth; «+» poor growth (OD_{540} 0.1-0.2); «++» moderate growth (OD_{540} 0.3—0.4); «+++» good growth (OD_{540} 0.4—0.5).

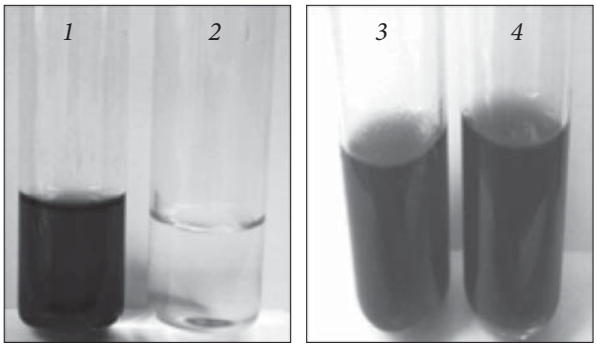

Fig. 1. Results of the Rother reaction to reveal the pathways for the benzene ring cleavage of monoaromatic substances. The purple color of the reaction mixture after growth of strain *R. aetherivorans* UCM Ac-602 on (*1*) benzene, (*3*) *toluene,* and (*4*) EX substances; (*2*) control without microorganisms

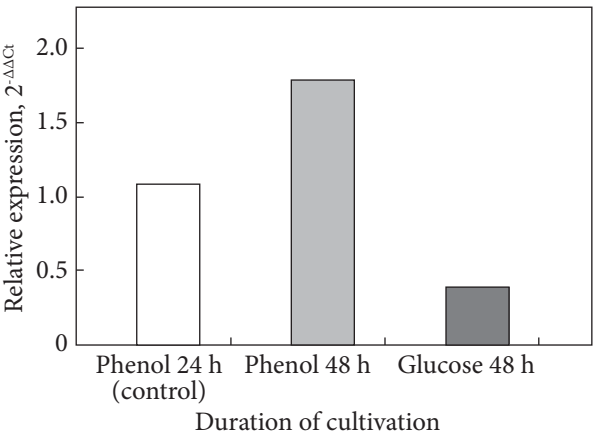

Fig. 2. The relative expression of the *catA* gene during cultivation of *R. aetherivorans* UCM Ac-602 with phenol or glucose. Gene expression after 24 h of growth with phenol is taken as 1 (control)

able to moderately utilize *para*-cresol. Strains *R. erythropolis* UCM Ac-51 and *G. rubripertincta* UCM Ac-125 moderately utilized *para*-nitrophenol, but *R. erythropolis* UCM Ac-29, UCM Ac-77, and UCM Ac-603 were unable to grow in the presence of this substrate. There was established good growth of *R. erythropolis* UCM Ac-603, moderate growth of *R. erythropolis* UCM Ac-29 and *R. aetherivorans* UCM Ac-602, and poor growth of *G. rubripertincta* UCM Ac-125 in the presence of resorcinol. Strains *R. erythropolis* UCM Ac-29, UCM Ac-51, and UCM Ac-603 as well as *R. aetherivorans* UCM Ac-602, and *G. rubripertincta* UCM Ac-125 were poorly able to use catechol. Phenol was well used by strains *R. erythropolis* UCM Ac-51, *R. aetherivorans* UCM Ac-602, and *G. rubripertincta* UCM Ac-125, but *R. erythropolis* UCM Ac-23 and UCM Ac-603 grew weakly in the presence of this substrate.

The analysis of the abilities of all tested strains to utilize monoaromatic compounds — derivatives of benzene and toluene — showed that this property is a strain-specific characteristic of the studied actinobacteria. Based on the obtained data, the profiles of the substrate specificity of actinobacteria toward monoaromatic compounds were established. It was shown that strain *R. aetherivorans* UCM Ac-602 with different intensities grew in the presence of the widest range of monoaromatic substances (12 out of 15 studied).

Possible ways of the benzene ring cleavage of aromatic compounds by actinobacteria were determined on the example of strain *R. aetherivorans* UCM Ac-602 under the conditions of its growth on benzene, toluene, EX, and BTX substances using the colorimetric Rotcher reaction. The dark purple coloration of the reaction mixture determined visually is indicative of an *ortho*- (β-ketoadipate) pathway cleavage of the aromatic ring (Fig. 1).

According to the analysis of literature data regarding the biodegradation pathways for monoaromatic compounds in microorganisms, genes encoding key enzymes of the initial stages of biodegradation of monoaromatic substances were selected for further evaluation of their relative transcriptional activity.

Verification of the specificity of the method and selected primers (*catA*-F and *catA*-R) to the sequence of the gene encoding catechol 1,2-dioxygenase, carried out using post-amplification analysis of melting curves of amplicons, showed that each sample was characterized by a single peak, which corresponds to a single amplification product and the absence of extraneous frag-

ments, such as primer dimers. To assess the *catA* gene expression activity, *R. aetherivorans* strain UCM Ac-602 was grown in a medium with phenol and glucose as a control substrate for 48 h. The results of quantitative PCR revealed that in comparison with the transcriptional activity of the *catA* gene at 24 h of growth on phenol taken as 1 (control), after 48 h the level of relative expression of the gene increased almost 2-fold on this substrate and 3-fold compared to glucose (Fig. 2). The obtained data indicate a high biodegradation potential of this strain in relation to monoaromatic substances and confirm the presence of the *ortho*-pathway for the cleavage of these substances, which is inherent in most *Rhodococcus* species (Krivoruchko et al., 2023).

Changes in the fatty acid composition of cells were studied on the example of *R. aetherivorans* UCM Ac-602 strain when it grows on monosubstrates — benzene, toluene, as well as BTX and EX substances. The obtained data were compared with the fatty acid composition of the cells of this strain during the assimilation of glucose. To interpret the obtained results, all identified fatty acids were divided into three groups: straight-chain saturated, straight-chain unsaturated, and methyl-branched fatty acids. It was established that the spectra of fatty acids during the growth of *R. aetherivorans* UCM Ac-602 on all studied substrates are almost identical (Table 2).

In cells grown on aromatic compounds and glucose, in the composition of fatty acids, straight-chain saturated hexadecanoic C16:0 (31.19—39.81 %), straight-chain unsaturated hexadecenoic C16:1 cis-9 (5.66—15.11 %), and octadecenoic C18:1 cis-9 (7.09—28.50 %) acids, as well as branched 10-methyloctadecanoic (tuberculostearic) (10Me-C18:0) (10.80—34.76 %) acid dominated. Depending on the substrate, the total amount of saturated acids ranged within 35.64—43.77 %, and unsaturated acids — within 19.07—42.97 % (Fig. 3).

The main difference in the fatty acid composition of cells during the assimilation of monoaromatic compounds compared to glucose was the content of individual acids (Table 2). Thus, cells grown on aromatic compounds contained 2.4—4.0 times less unsaturated C18:1 cis-9 fatty acid and 2.8—3.2 times more methyl-branched 10Me-C18:0 acid compared to the growth on glucose. In

Table 2. **Fatty acid composition of cells of *R. aetherivorans* strain UCM Ac-602 during growth on benzene, toluene, BTX and EX substances, and glucose**

Fatty acids	Percentage of total fatty acids				
	Benzene	Toluene	BTX	EX	Glucose*
$C_{14:0}$	0.81±0.06	0.69±0.04	0.73±0.05	—	1.30±0.07
$C_{15:0}$	0.67±0.04	0.46±0.02	0.50±0.03	—	1.58±0.11
$C_{16:1}$ *cis*-9	13.58±0.85	14.21±0.98	15.11±1.06	5.66±0.35	14.47±1.01
$C_{16:0}$	33.79±2.36	34.58±2.41	32.73±1,96	39.81±2.39	31.19±1.88
14Me-$C_{16:0}$	—	—	0.32±0.02	—	—
$C_{17:0}$	1.34±0.08	0.93±0.06	0.99±0.07	—	4.36±0.31
$C_{18:1}$ *cis*-9	9.98±0.60	9.54±0.57	11.83±0.81	7.09±0.35	28.50±1.71
$C_{18:0}$	0.74±0.05	0.53±0.04	0.69±0.05	3.14±0.19	4.38±0.26
10Me-$C_{18:0}$	33.48±2.00	34.76±2.43	32.09±2.25	29.88±1.79	10.80±0.65

* data are given in (Nogina et al., 2021); BTX — a mixture in equal proportions of benzene, toluene, and *ortho*-xylene; EX — a mixture in equal proportions of ethylbenzene, *ortho*- *meta*- and *para*-xylene. The amount of acids is presented as a percentage of the average of 3 measurements; «—» no acid detected or its amount is less than 0.3%. The standard deviation did not exceed 7% of a particular value.

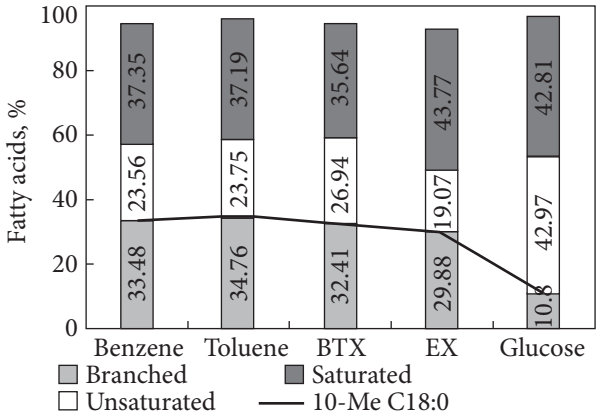

Fig. 3. The total amount of branched, straight-chain saturated and unsaturated fatty acids in the cells of the *R. aetherivorans* U**C**M Ac-602 strain during growth on different substrates: BTX — a mixture of benzene, toluene, and *ortho*-xylene; EX — a mixture of ethylbenzene and *ortho*-, *meta*- and *para*-xylene

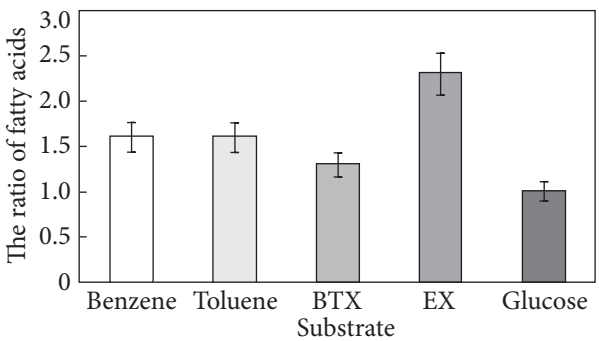

Fig. 4. The ratio of straight-chain saturated and unsaturated fatty acids of the *R. aetherivorans* strain U**C**M Ac-602 during growth on different substrates: BTX — a mixture of benzene, toluene, and *ortho*-xylene; EX — a mixture of ethylbenzene and *ortho*-, *meta*- and *para*-xylene

addition, when the strain grew on monoaromatic compounds, significant changes were observed compared to glucose in the ratio of saturated and unsaturated fatty acids in the cells. Determination of the ratio in strain *R. aetherivorans* UCM Ac-602 during growth on aromatic compounds showed an increase in this parameter compared to glucose by 1.3—2.3 times (Fig. 4).

Discussion. Among monoaromatic substances, the main water pollutants, especially groundwater, are BTEX compounds (benzene, toluene, ethylbenzene, and xylene isomers). They are part of the aromatic components of gasoline and enter the environment as a result of leaks from underground tanks, pipelines, accidental spills, causing serious environmental problems (El-Naas et al., 2014). According to literature data, the bacteria of the genus *Rhodococcus* and representatives of other taxonomically related genera of actinobacteria are important members of the bacterial communities of aquatic and terrestrial ecosystems polluted with aromatic substances (Song et al., 2011; Alvarez et al., 2017). The role of these microorganisms in the conversion of many highly oxidizing and toxic organic substances, such as aliphatic and aromatic compounds, has been well studied (Larkin et al., 2010; Nazari et al., 2022; Behera & Das, 2023). Most currently known actinobacteria — destructors of monoaromatic substances can assimilate only certain components of BTEX. It is known that among BTEX substances, *ortho*-xylene is the most difficult compound for biodegradation by microorganisms, although some representatives of the genus *Rhodococcus* (*Rhodococcus* sp. BTO62) are capable of slowly utilizing it (Jeong & Shoda, 2008). Some strains of the genus *Rhodococcus* are able to use both *ortho*-xylene and benzotriazole (Haroune et al., 2002; Di Canito et al., 2018). There are data in the literature that strains of *Rhodococcus* sp. EH831 (Lee et al., 2010), *Rhodococcus* sp. EC1 (Lee & Cho, 2008), *Rhodococcus wratislaviensis* IFP2016 (Auffret et al., 2015), *Rhodococcus rhodochrous* TRN7 (Rodrigues et al., 2015), and *Rhodococcus* sp. ZJUT312 (You et al., 2018) can assimilate all BTEX monocompounds. The described strains of *Rhodococcus* sp. DK17 (Kim et al., 2002; Kim et al., 2007) and *Rhodococcus opacus* R7 (Di Gennaro et al., 2001) assimilate benzene, toluene, ethylbenzene, and ortho-xylene but not *meta*- and *para*-xylenes. The *R. aetherivorans* IFP2017 strain (Auffret et al., 2015) was found to be able to grow only on benzene, in contrast to *R. aetherivorans* BCP1, which assimilated all BTEX substances, as well

as naphthalene and phenol (Orro et al., 2015). These data indicate that the ability to biodegrade monoaromatic substances in actinobacteria is a strain-specific characteristic.

In this study, we have determined the ability of active strains of hydrocarbon-oxidizing actinobacteria from the Ukrainian Collection of Microorganisms of the IMV of NASU belonging to the species *R. erythropolis*, *R. aetherivorans*, *D. maris,* and *G. rubripertincta* to assimilate monoaromatic compounds — derivatives of benzene (including BTEX substances) and phenol. Strain *R. aetherivorans* UCM Ac-602 studied in our work, previously described as an active phenol destructor (Nogina et al., 2020) had the widest spectrum of substrate specificity toward monoaromatic substances: it utilized 12 out of 15 studied compounds, including benzene, toluene, and *ortho*-xylene. A slightly smaller spectrum of substrate specificity toward monoaromatic compounds was found in strain *G. rubripertincta* UCM Ac-125, which assimilated 11 out of 15 studied substances and, unlike *R. aetherivorans* UCM Ac-602, utilized benzotriazole, but not *ortho*-xylene and *meta*-cresol. Strain *R. erythropolis* UCM Ac-51 is close to the specified strains in terms of their ability to assimilate a significant number (9 from 15) of the studied monoaromatic compounds. The least number of studied compounds (4 from 15) was assimilated by strain *D. maris* UCM Ac-205. The analysis of obtained results confirmed that representatives of various species of actinobacteria are able to use different monoaromatic compounds as growth substrates. However, they do not have a strict correlation between the taxonomic position and the ability to metabolize these substances (Krivoruchko et al., 2023).

It is well known that the most important stage of biodegradation of monoaromatic substances is the cleavage of the benzene ring. In general, aromatic pollutants are resistant to microbial degradation due to the inherent thermodynamic stability of the benzene ring. However, some microorganisms, in particular actinobacteria of the genus *Rhodococcus*, developed a wide range of catabolic enzymatic pathways for the biodegradation of aromatic pollutants. Members of this genus are often isolated from aromatic hydrocarbons contaminating environments and used widely in bioremediation or in bioengineering processes due to their catabolic diversity (Panigrahy et al., 2022). A central intermediate in the degradation pathways of many monoaromatic compounds is catechol. Aromatic ring-cleavage enzymes, such as catechol1,2-dioxygenase (key enzyme of *ortho*-cleavage pathway), which is encoded by the *catA* gene, play a central role in the metabolism of aromatic compounds by *Rhodococcus* species. The presence of the *catA* gene in microorganisms indicates that they are able to assimilate monoaromatic substances through the *ortho*-pathway of catechol cleavage (Táncsics et al., 2008, Krivoruchko et al., 2023). Only in a small amount of rhodococci species, are found the key enzyme of *meta*-pathway of catechol cleavage, catechol 2,3-dioxygenases. So, the results of the study of 133 rhodococci strains belonging to the species *R. aetherivorans*, *R. erythropolis*, *R. qingshengii*, *R. cerastii*, *R. corynebacterioides*, *R. fascians*, *R. globerulus*, *R. jostii*, *R. opacus*, *R. pyridinivorans*, *R. rhodochrous*, *R. ruber*, *R. wratislaviensis* and *R. yunnanensis* have shown that the preferred pathway for catechol cleavage was *ortho*-oxidation, as catechol 1,2-dioxygenases were revealed in all studied genomes. Catechol 2,3-dioxygenases were found only in *R. opacus* IEGM 249, *R. pyridinivorans* IEGM 1137, and *R. ruber* IEGM 231 genomes (Krivoruchko et al., 2023).

There are described *Rhodococcus* strains, in particular *Rhodococcus* sp. DK17, which possesses two different ring-cleavage pathways of the biodegradation of aromatic compounds, although the initial oxidation reactions may be catalysed by a common oxygenase. It was established that the *ortho*-cleavage pathway in this strain is specifically induced by benzene rather than by *ortho*-xylene. Strain *Rhodococcus* sp. DK17 can lose the *meta*-cleavage activity as a result of the loss

of the key enzyme (catechol 2,3-dioxygenase) coding large linear plasmids. The loss of plasmid does not influence the *ortho*-cleavage activity; therefore, it can be assumed that the *ortho*-cleavage key enzyme, the catechol 1,2-dioxygenase, is coded on the chromosome (Kim et al., 2002).

One of the simplest methods for determination of pathways of aromatic compound biodegradation by microorganisms is the Rother colorimetric reaction. Development of a dark purple color in the reaction mixture indicates the presence of β-ketoadipic acid, which is one of the initial intermediates of the *ortho*-pathway of biodegradation of monoaromatic substances. The Rothera test results showed that the *R. aetherivorans* UCM Ac-602 strain could degrade benzene, toluene, BTX and EX by the *ortho*-cleavage (β-ketoadipate) pathway because there was observed the development of a dark purple color of the *ortho*-cleavage product. It is noteworthy that the *ortho*-cleavage pathway is frequently chromosomally encoded and is widely distributed in soil bacteria and fungi, constituting the major pathway for aromatic compounds catabolism in these organisms (Erdoğmuş et al., 2013). A variety of *catA* genes are distributed to a large extent in microorganisms that metabolize aromatic compounds through the *ortho*-cleavage pathway (Matsumura et al., 2004, Matsumura et al., 2006). Expression of the *cat*A gene detected in *R. aetherivorans* UCM Ac-602 during growth on phenol as well as on glucose suggests that this strain is capable of constitutively synthesizing catechol 1,2-dioxygenase. Our results are in good agreement with the information on other representatives of the genus *Rhodococcus*. So, it was shown that the *Rhodococcus* sp. AN-22 strain, which degrades aniline (methyl benzene) by *ortho*-cleavage and synthesizes catechol 1,2-dioxygenase constitutively, produces this enzyme when assimilating not only aniline but also 21 non-aromatic substrates, in particular D-glucose. In contrast, aniline-assimilating strains of *Rhodococcus erythropolis* AN-13 and *Frateuria* sp. ANA-18, which synthesized catechol 1,2-dioxygenase, did not produce the enzyme in the absence of aniline (Matsumura et al., 2004). The significant increase in the expression level of the *catA* gene in the process of phenol assimilation by *R. aetherivorans* UCM Ac-602 testifies to the high metabolic ability of this strain to biodegrade aromatic substances by the *ortho*-pathway. Additional research is required for a more detailed analysis of the *catA* gene coding catechol 1,2-dioxygenase to provide more definitive information about the features of this enzyme synthesis in the *R. aetherivorans* UCM Ac-602 strain.

It is known that the cell membrane lipids play an important role in the adaptation of microorganisms to changing environmental conditions. Fatty acids, which are the main components of membrane lipids, primarily contact and react to all changes in the environment, maintaining the relationship between the structure and function of the membrane. One of the mechanisms of adaptation of microorganisms to the toxic effect of monoaromatic substances is an increase in the degree of saturation of fatty acids in cell membranes. It was established that an increase in the number of saturated acids makes the membrane less permeable to toxic substances, as these acids contribute to increasing the rigidity of the membrane due to the continuous packing of their acyl chains (Chang & Cronan,1999; Konings et al., 2002; De Carvalho, 2019; Rodriges & de Carvalho, 2019). We found that, in comparison with glucose, in the *R. aetherivorans* UCM Ac-602 strain, in the process of assimilation of benzene, toluene, BTX and EX substances, the amount of the main unsaturated fatty acid in the cells (C18:1 cis 9) significantly decreases, whereas the amount of the main methyl-branching acids (10Me-C18:0) and the ratio of saturated to unsaturated acids increases. The obtained results are consistent with our previous studies of changes in the fatty acid composition of cells of this strain during phenol assimilation (Nogina et al., 2021). Similar data were described in the literature for other species of rho-

dococci when they grow on monoaromatic compounds. Thus, according to Tsitko et al. (Tsitko et al., 1999), in *R. opacus* strains, during assimilation of phenol and toluene, the content of 10-methyl branched fatty acids in cells was 3—10 times higher than in cells grown on fructose. It is known that an increase in the content of branched-chain fatty acids in cell membranes, in particular 10Me-18:0 acid, can stabilize the gel phase of membrane bilayers, which contributes to an increase in membrane rigidity and a decrease in the diffusion of toxic substances through it (Lindström et al., 2006). The changes in the fatty acid composition of cells that we established during the assimilation of benzene, toluene, BTX, and EX substances by strain *R. aetherivorans* UCM Ac-602 may indicate the participation of the detected fatty acids in the adaptation of the cells of this strain to the assimilation of aromatic compounds.

Thus, it was found that the studied in this work hydrocarbon-oxidizing strains of actinobacteria belonging to the species *R. erythropolis*, *R. aetherivorans*, *D. maris*, and *G. rubripertincta* differ in their ability to assimilate monocyclic aromatic substances — derivatives of benzene and phenol. The widest range of monoaromatic substances (80 % from all analyzed) was used by strain *R. aetherivorans* UCM Ac-602. The biodegradation of monoaromatic substances by this strain occurs through the *ortho*-pathway of benzene ring cleavage. One of the possible ways to adapt *R. aetherivorans* UCM Ac-602 to the assimilation of monoaromatic compounds is to change the lipid composition of cells, in particular, to reach a significant decrease (by 2.4—4.0 times) in the amount of unsaturated acids and an increase (by 2.8—3.2 times) in the amount of methyl-branched acids, which increases the rigidity of the membranes and reduces their permeability to a toxic substrate. The obtained data indicate that the *R. aetherivorans* UCM Ac-602 strain is promising for use in biotechnologies for purification the environment from hazardous aromatic ecotoxicants.

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БІОДЕСТРУКЦІЯ МОНОАРОМАТИЧНИХ РЕЧОВИН ВУГЛЕВОДЕНЬОКИСНЮВАЛЬНИМИ АКТИНОБАКТЕРІЯМИ

Моноароматичні речовини належать до найбільш поширених і особливо небезпечних забруднювачів довкілля. Вони є компонентами стічних вод нафтопереробних заводів, вугільної та хімічної промисловості і часто зустрічаються в ґрунті, підземних і поверхневих водах. Використання актинобактерій для біоремедіації забруднених цими речовинами природних і промислових об'єктів є екологічно та економічно вигідною альтернативою фізико-хімічних методів очищення. Тому актуальним є пошук нових штамів актинобактерій, здатних засвоювати моноароматичні сполуки, для подальшого використання їх у біотехнологіях очищення забрудненого цими речовинами середовища. **Мета.** Визначити здатність вуглеводеньокиснювальних штамів актинобактерій Української колекції мікроорганізмів до засвоєння моноароматичних сполук — похідних бензолу та фенолу. **Методи.** Культивування штамів проводили на рідкому мінеральному середовищі за початкової концентрації моноароматичних сполук 500 мг/л як єдиного джерела вуглецю та енергії. Оцінку активності експресії гена *catA*, що кодує катехол 1,2-диоксигеназу — ключовий фермент початкового етапу біодеструкції моноароматичних сполук, проводили за умов росту культур на фенолі і глюкозі. Відносну експресію гена *catA* оцінювали методом ЗТ-кПЛР. Метилові ефіри жирних кислот отримували гідролізом клітин у 5 % розчині ацетилхлориду в метанолі з наступною екстракцією сумішшю ефір-гексан. Ідентифікацію метилових ефірів проводили за допомогою хромато-мас-спектрометричної системи Agilent 6800N/5973 inert (Agilent Technologies, US). Вміст жирних кислот визначали за допомогою програмного забезпечення AgilentChemStation. **Результати.** Встановлено, що досліджувані штами актинобактерій, які належать до видів *Dietzia maris*, *Gordonia rubripertincta*, *Rhodococcus aetherivorans* та *Rhodococcus erythropolis,* відрізняються між собою за здатністю засвоювати моноциклічні ароматичні сполуки — похідні бензолу та фенолу. Більшість штамів краще росли на середовищах з бензолом та його похідними. Усі штами добре росли на суміші етилбензолу та *орто*-, *мета*- і *пара*-ксилолу, більшість з них з різною інтенсивністю росли на суміші бензолу, толуолу та *орто*-ксилолу (БТК) та на моносубстратах — бензолі, бензотриазолі і бензоаті. Толуол використовували 50 % досліджуваних штамів, і лише один з них (*R. aetherivorans* УКМ Ас-602) ріс на *орто*-ксилолі. Менша кількість штамів росла на середовищах з похідними фенолу. Вони не засвоювали *орто*-крезол і гідрохінон, але з різною інтенсивністю росли на фенолі, паранітрофенолі, резорцині і катехолі. Лише декілька штамів росли в присутності *мета*- та *пара*-крезолу. Найбільш широкий спектр моноароматичних речовин засвоював штам *R. aetherivorans* УКМ Ас-602. Показано, що в процесі засвоєння цим штамом фенолу рівень транскрипційної активності гену *catA*, що кодує ключовий фермент *орто*-шляху розщеплення ароматичного кільця — катехол 1,2-диоксигеназу, збільшувався майже у 2 рази через 48 год порівняно з 24 год росту штаму на цьому субстраті та у 3 рази — порівняно з глюкозою. Отримані дані свідчать про здатність штаму *R. aetherivorans* УКМ Ас-602 засвоювати моноароматичні речовини шляхом *орто*-розщеплення. У процесі росту на бензолі, толуолі, БТК та ЕК речовинах у пулі жирних кислот клітин цього штаму переважали гексадеканова С16:0 (32,73—39,81 %), гексадеценова С16:1 цис-9 (5,66—15,11 %) та октадеценова С18:1 цис-9 (7,09—11,83 %) кислоти, а також 10-метилоктадеканова 10-Ме-С18:0 (29,88—34,76 %) кислота. У складі жирних кислот клітин, вирощених на ароматичних сполуках, на відміну від вирощених на глюкозі, містилося у 2,4—4,0 рази менше ненасиченої С18:1 цис-9 кислоти і в 2,8—3,2 рази більше метилрозгалуженої кислоти 10Ме-С18:0. **Висновки.** Засвоєння моноароматичних сполук — похідних бензолу та фенолу — є штам-специфічною ознакою видів актинобактерій. На прикладі штаму *R. aetherivorans* УКМ Ас-602 показано, що засвоєння моноароматичних сполук відбувається у досліджених актинобактерій *орто*-шляхом. Одним із механізмів адаптації штаму *R. aetherivorans* UCM Ac-602 до засвоєння цих речовин є значне зниження вмісту в клітинах ненасичених жирних кислот та збільшення кількості метил розгалужених кислот, що сприяє підвищенню жорсткості мембран і стійкості клітин до токсичної дії цих субстратів. Отримані дані свідчать про високий рівень адаптації актинобактерій до засвоєння моноароматичних сполук та перспективність подальшого вивчення їх з метою використання в біотехнологіях очищення навколишнього середовища від цих речовин-забрудників.

Ключові слова: актинобактерії, *Dietzia, Gordonia, Rhodococcus, моноароматичні речовини, жирні кислоти, ген catA, катехол 1,2-діоксигеназа.*