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POLYPHASIC TAXONOMY ANALYSE OF *COMAMONAS TESTOSTERONI* RESISTANT TO HEXACHLOROBENZENE

Mariia Dimova, Liudmyla Dankevych, Nadiya Yamborko, Galyna Iutynska*

Address(es): Mariia Dimova,
Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Department of General and Soil Microbiology, 154 Acad. Zabolotny str., 03143, Kyiv, Ukraine, phone:+38(096) 844 97 65.

*Corresponding author: mdildiv@gmail.com

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ABSTRACT

Based on the analysing phenotypic and genotypic properties, the taxonomic position of isolates # 46 and # 47 was established and assigned to the species *Comamonas testosteroni* UCM B-400 and UCM B-401. Isolates were selected from the soil with organochlorine wastes landfill of chemical enterprises in Kalush, Ivano-Frankivsk region, Ukraine. The strains are resistant to high hexachlorobenzene concentrations. According to morphological, cultural and biochemical properties, the studied strains are related to typical members of the *Comamonadaceae* family, they are motile gram-negative rods, aerobic, chemoorganotrophic. Saturated and unsaturated fatty acids with a carbon chain length from C10 to C18 were found in composition of cellular lipids. The qualitative and quantitative composition of marker hydroxy acids, namely: 2 hydroxyhexadecane ($C_{16:\ 0\ 2OH}$) and 3 hydroxydecane ($C_{10:\ 0\ 3OH}$) in the lipids of the strains ranged from 2-5%, which corresponded to their content in the representatives of the *Comamonas* genus. Phylogenetic analysing the nucleotide sequences of the 16S rRNA gene revealed the affinity of isolates # 46 (97.77%) and # 47 (98.05%) with a similar nucleotide sequence of a typical strain of *Comamonas testosteron*i LMG 1800. The 16S rRNA nucleotide sequences of *Comamonas testosteroni* UCM B-400 and UCM B-401 were placed in GenBank under the numbers SUB9376895 Comamonas_testosteroni_46 MW861636 and SUB9376895 Comamonas_testosteroni_47 MW861637.

Keywords: resistance to hexachlorobenzene, polyphasic taxonomy analyse, *Comamonas testosteroni*

INTRODUCTION

Bioremediation as a way to purify ecotopes from xenobiotics is becoming increasingly popular, due to cost-effectiveness and easing use. There are various bioremediation methods, one of which is "bioaugmentation", which involves the introduction into the contaminated environment of microorganisms capable to destroy the target pollutans (**Ghosal, 2016**). Betaproteobacteria are known to be widespread in soil ecosystems. Also *Betaproteobacteria* are reported to be able to destruct chloroaromatic, nitroaromatic and aminoaromatic compounds under aerobic conditions. (**Tan *et al.*, 2019**). Studying microbial communities of xenobiotic-contaminated soils showed the members of the genera *Pseudomonas* and *Comamonas* to be often dominate in the microbiome. The data about ability of *P. maltophilia, P. putida* and *C. testosteroni* to destroy benzene and xylene are presented in literature (**Mallakin *et al.* , 1996**). The more higher metabolic activity of *Comamonas testosteroni* in combination with *Bacillus subtilis* DKT to the destroying chlorobenzenes and toluenes has been studied and shown (**Nguyen, *et. al.*, 2019**). In the species composition of bacterial communities from river sediments contaminated with nitrobenzene, members of families *Comamonadaceae* and *Clostridiaceae* were dominant giving the authors reason to believe about their participation in the pollutants biodegradation (**Li *et al.*, 2009**). In the pesticide-contaminated agricultural soil 11 bacterial genera were dominated: *Pseudomonas, Bacillus, Acinetobacter, Flavobacterium, Comamonas, Achromobacter, Rhodococcus, Ochrobactrum, Aquamicrobium, Bordetella and Microbacterium* (**Hassen *et al.*, 2017**). Tolerancing these bacteria to xenobiotics allows to make an assumption about the potential for destruction these toxic substances and assimilate its as carbon and energy sources. Sun and co-authors noted that C. testosteroni inoculating into soil contaminated with polycyclic aromatic hydrocarbons (PAHs) increased microbial activity to PAHs metabolism by changing the the microbiocenosis structure and enhancing the interaction between bacteria capable to PAHs decomposing (**Sun *et al.*, 2021**). Thus, members of the genus *Comamonas* spp. are promising for use in bioremediation of contaminated areas. The actual problem is to isolate new bacterial strains from different surroundings for their possible use in the biological remediation processes of soil. Isolating of new bacterial strains as xenobiotic destructors involves not only the studying its ability to destroy certain pollutants, but also to determine their taxonomic status. Based on this, the aim of our work obtaining hexachlorobenzene-resistant isolates and its identification using polyphase analysis. Studying phenotypic and genotypic properties complex was carried out to determine the systematic position of two isolates # 46 and # 47, selected from the organochlorine waste landfill in Kalush.

MATERIALS AND METHODS

Soil sampling and selection of bacterial isolates

Soil samples were taken from the surface layer (2-50 cm) of the toxic waste landfill from the chemical concern "Oriana-Galev" near the city of Kalush, Ivano-Frankivsk region, Ukraine. Screening of isolates resistante to hexachlorobenzene (HCB) was conducted by culturing its in Menkina's mineral medium of the following composition per l: $NaNO_3$ - 2, KCl - 0.6, 10% $MgSO_4$ - 5 ml, 50% glucose - 10 ml, 20% K_2HPO_4 - 15 ml. HCB dose by 50 mg*l^{-1} was added to the medium. Microbial isolates were separated by sowing the soil suspension on agar nutrient medium M17 (Oxoid Ltd, United Kingdom) containing HCB in concentrations from 100 to 500 mg*l^{-1}. (**Public Health England, 2019**).

Microorganisms

Isolates #46 and #47 were selected as single colonies grown on agar nutrient medium M17 (Oxoid Ltd, United Kingdom) by sowing suspensions of soil samples from the landfill of toxic waste, mainly hexachlorobenzene. (the city of Kalush, Ivano-Frankivsk region of Ukraine). The typical strains from the Ukrainian Collection of Microorganisms *Pseudomonas putida* UCM B-115, *Comamonas testosteroni* UCM B-213, *C. terrigena* UCM B-363, and *Delftia acidovorans* UCM B-197 were used as standards.

Determination of bacterial resistance to HCB

Mineral Menkina's medium contained 50 mg*l^{-1} of HCB was inoculated by 1-day culture of the studied isolates with a titer of 3.9 *10^9 (# 46) or 4*10^7 (# 47) colonies forming units (CFU) per ml. Its were incubated for 168 hours.

Determination of the final cells titer was perfomed by sowing ten-fold dilutions of suspensions on agar medium M17 followed by counting CFU, which corresponded to the quantity of living cells in the suspension. The titer of cells was expressed by cells*ml^{-1}.

Analysis of morphological, cultural, physiological and biochemical properties

The morphology of the studied bacteria was examined by light microscopy of Gram-stained 1-day cultures grown on agar nutrient medium M17 (Oxoid Ltd, United Kingdom). Physiological and biochemical properties and primary identification of isolates were carried out using API test systems 20E bioMérieux (France). Studying biochemical properties of isolates was also conducted by comparative analysis with typical strains from the Ukrainian Collection of Microorganisms: *D. acidovorans* UCM B-197, *C. testosteroni* UCM B-213, *P. putida* UCM B-115 in the automated system VITEK® 2 System bioMérieux (France) according to the manufacturer's instructions using the VITEK® 2: Gram-Negative identification card (GN), designed to identify gram-negative rods.

Fatty acid analysis

The fatty acid spectrum of total cellular lipids was determined by the analysing methyl esters method. For chemotaxonomic analysis, the bacteria were grown on potato glucose agar at 28 ºC for 24 hours. Cell hydrolysis was performed in 5% acetyl chloride solution in methanol for one hour at 100 ºC, followed by extraction with ether-hexane (1: 1) mixture. Fatty acid methyl esters identification was performed using a chromato-mass spectrometric system Agilent 6800N / 5973 inert. (Aglient Technologies, USA). Methyl esters were automatically identified by its retention time compared to the synthetic standards (Supelco Analytical Products, USA). The fatty acid composition was determined using Agilent masshunter workstation software ver.10.0.368 and displayed as a percentage of the total peak area (**Patika V.P. et al., 2017**).

DNA isolation

Bacterial DNA was extracted by the express method. To do this, 1 ml of cell suspension was centrifuged at 13.5 thousand rpm on a Minispin centrifuge (Eppendorf) for 2 min and the supernatant was removed. The bacterial pellet were resuspended in 200 µl of TE buffer and incubated in a thermostat at 95 °C for 5 minutes. Cell debris was precipitated by centrifugation at 5.0 thousand rpm for 2 min and 180–190 µl of supernatant were gathered, 2 µl of which were used for polymerase chain reaction (PCR).

16S rRNA gene sequencing analysis

The 16S rRNA gene was amplified using universal bacterial primers pA-5'-AGAGTTTGATCCTGGCTCAG-3 ' (8-27, *E. coli* numbering) and pH-3'-AAGGAGGTGATCCAGCCGCA-5' (1542-1523, *E. coli* numbering) (Edwards U., 1989). The PCR mixture, with a total volume of 25 µl, contained: 2 µl of bacterial DNA; 1 x DreamTaq ™ Green Buffer (Thermo Fisher Scientific); 0.4 mM each dNTP; 0.4 µM of each primer and 1U DreamTaq DNA Polymerase (Thermo Fisher Scientific). Amplification was carried out using a thermal cycler 2720 Thermal Cycler (Applied Biosystems, USA) under experimentally selected conditions (35 cycles): initial denaturation 95 °C - 3 min; basic denaturation - 95 °C / 30 s., annealing of primers - 59 °C / 25 s., elongation - 72 °C / 1 min. 30s.) and the final DNA synthesis - 72 °C / 2 min. The amplificates presence in the samples was determined by electrophoresis in 1xTAE buffer and 1% agarose gel with the additing ethidium bromide solution. The fragment was excised from an agarose gel and DNA was isolated using Silica Bead DNA Gel Extraction Kit (ThermoFisher Scientific, Lithuania). The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). 16S rRNA gen were sequenced from the 5 'and 3' ends on an automatic sequencer 3130 Genetic Analyzer (Applied Biosystems / HITACHI) using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequences of strain # 46 with 1437 and strain # 47 with 1487 nucleotides were obtained.

Statistical data analysis

The constructing the complementary sequence was performed using the program Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/). Editing and alignmenting sequences were performed using the program Multalin (http://multalin.toulouse.inra.fr/multalin/). Determinating affinity of 16S rRNA gene nucleotide sequences of the studied strains with homologous nucleotide sequences of typical strains of the families *Comamonadaceae*, *Burkholdereaceae* and *Pseudomonadaceae*, placed in GenBank, was performed using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The MEGA 5 program was used to construct the phylogenetic relationships dendrogram (**Tamura *et al*., 2011**). The dendrograms were inferred applying the Neighbor Joining method using Kimura's two-parameter model based on 100 replicates of the "bootstrap" assay.

RESULTS AND DISCUSSION

HCB content in the studied soil

At the Oriana-Galev chemical factory, the producing organochlorine compounds: carbon tetrachloride and perchlorethylene were carried out by direct chlorinating industrial hydrocarbon raw materials, mainly methane. The result of these technological processes was the formation of solid toxic waste in the amount of 540 tons per year with a content of hexachlorobenzene (HCB) over 90%. At different distances from the landfill, the HCB content in the soil layer 0-50 cm ranged from 38.50 to 3913.00 mg / kg, which exceeded the maximum permissible concentrations (MPC = 0.03 mg / kg) in 1283 - 130433 times **(Hertsyuk, 2012)**. These wastes belong to the first class of danger. Hexachlorobenzene according to the Stockholm Convention (http://www.pops.int/TheConvention/Overview/TextoftheConvention/tabid/2232/Default.aspx) classified as persistent organic pollutants, it is a dioxin-like toxicant with carcinogenic, teratogenic, immunotoxic properties, irritating the mucous membrane and skin **(Devi, 2020)**.

Resistance of bacterial isolates to HCB

More than 60 isolates were isolated on agar medium M17 containing different HCB doses, among which isolates # 46 and # 47 were selected for further study as the most resistant to HCB contamination. The studying the resistance of the selected isolates to HCB showed to be able to grow in Menkina's liquid medium with the presence of high dose 50 mg*l^{-1} HCB, as corresponded to 1660 MPC. After cultivating for168 hours in medium containing 50 mg* l^{-1} HCB, the cell titers were for isolate # 46 - 8.8*10^{6} and isolate # 47 - 1.2*10^{6} cells*ml^{-1}. The initial titers as the medium was inoculated were for isolates # 46 and # 47 - 3.9 * 10^{9} and - 4 * 10^{7} cells*ml^{-1} , respectively.

Morphological and chemotaxonomic properties of isolates

According to morphologycal properties, isolates # 46 and # 47 are aerobic gram-negative rods with sizes 0.4 × 2.1 µm and 0.3 × 2.1 µm, respectively. Studying selected isolates were performed as comparisying to the collection strains of *P. putida* UCM B-115, *C. testosteroni* UCM B-213 and *D. acidovorans* UCM B-197. By cell morphology, its are non-spore-forming, aerobic, gram-negative rods with polar flagella. All strains are hemoorganotrophs, catalase-positive and oxidase-positive. Both the collecting and isolated strains previously belonged to the family *Pseudomonadaceae*. The classification of these genera changed, as a resulting from the genus *Pseudomonas* as independent genera *Comamonas* and *Delftia* were separated. The primary establishing physiological and biochemical characteristics was performed using API test systems 20E bioMérieux, as resulted relating isolates # 46 and # 47 to typical members of the family *Comamonadaceae* (Table 1).
Further identification was performed in the automated Vitek 2 BioMérieux system, as resulted established the relating isolate # 46 to *Delftia acidovorans* (affinity 95%) and isolate # 47 to *Comamonas testosteroni* (affinity 99%). Simultaneously with the studied strains, comparative researching as control typical strains of *Comamonas testosteroni* UCM B-213 and *Delftia acidovorans* UCM B-197 from the Ukrainian Collection of Microorganisms, was performed in the automated system Vitek 2 System.

Analysing test results using Vitek 2 System Bio-Mérieux, based on the interacting the studied strains with differentiating substrates allowed to determine for each strain the authenticity markers needed to establish its belonging to certain microbial species. The following authenticity markers for *Delftia acidovorans* UCM B-197 were established : assimilation of lactate and malate, alkalization of succinate, fermentation of elman, as well as enzymatic activities: glutamylarylamidase, pyrrolidone-arylamidase, tyrosinarylamidase, tyrosinarylamidase. Characteristic features for *Comamonas testosteroni* UCM B-213 were the ability to alkalize lactate and succinate, tyrosinarylamidase, pyrrolidone-arylamidase activity. Both collection strains didn't assimilate glucose, lactose, trehalose and other sugars, didn't utilize malonate, sodium citrate, didn't produce hydrogen sulfide. Studied isolates # 46 and # 47 were not able to assimilate most sugars, did not produce H2S, did not reduce nitrates to molecular nitrogen, did not hydrolyze gelatin, but were able to assimilate organic acids such as lactate and amino acids.
The obtained results showed the identity of newly selected isolate # 47 to the typical strain of *Comamonas testosteroni* UCM B-213 by 99%, and isolate # 46 - to *Delftia acidovorans* UCM B-197 by 95%. Due to the significant affinity of morphology-cultural and physiology-biochemical characteristics of *Comamonas testosteroni* and *Delftia acidovorans*, these properties alone are not enough to correctly identify selected strains to the species, so we determined other chemotaxonomic traits, in particular, the fatty acids composition of cell lipids.

Table 1 Phenotypic properties of the isolates compared with closely related typical collection strains

Reaction/enzymes	*Pseudomonas putida* UCM B-115	*Delftia acidovorans* UCM B-197	*Comamonas testosteroni* UCM B-213	Isolate #46	Isolate #47
β-galoctosidase	-	-	-	-	-
Arginindihydrolase	+	-	-	-	-
Lisinedecarboxylase	-	-	-	-	-
Ornithinedecarboxylase	-	-	-	-	-
Citrate utalisation	+	-	-	-	-
H_2S production	-	-	-	-	-
Urease	-	-	-	-	-
Tryptophanedeaminase	-	+	+	+	+
Indole production	-	-	-	-	-
Acetoine production	-	+	+	+	+
Gelatinase	-	-	-	-	-
D-glucose fermentation/oxidation	+	-	-	-	-
D-mannitol fermentation/oxidation	-	-	-	-	-
Inositol fermentation/oxidation	-	-	-	-	-
D-sorbitol fermentation/oxidation	-	-	-	-	-
L-rhamnose	-	-	-	-	-
D-saccharose	-	-	-	-	-
D-melibiose	-	-	-	-	-
Amygdaline	-	-	-	-	-
L-arabinose	-	-	-	-	-
NO_2 production	-	-	+	+	+
N_2 production	+	+	-	-	-

Legend: «+» – positive reaction; «-» – negative reaction

The fatty acids composition of total cellular lipids

The fatty acids composition of total cellular lipids is important for determining the taxonomic bacterial position. In the cellular lipids of the studied isolates # 46 and # 47, fatty acids with a carbon chain length from C10 to C18 were detected, namely: unsaturated - hexadecenoic (C16: 1cis 9) and cis-9 octadecenoic acid (C18: 1 cis 9); saturated - dodecane (C12:0), tetradecane (C14:0), pentadecane (C15:0), hexadecane (C16:0), heptadecane (C17: 0), octadecanoic (C18: 0) acids; hydroxy acids - 2 hydroxyhexadecanoic (C16:0 2OH) and 3 hydroxydecanoic (C10:0 3OH) acids. Unsaturated hexadecenoic (C16: 1cis 9) and cis-9 octadecenoic acids (C18: 1 cis 9) and saturated hexadecanoic (C16: 0) acids were dominant in the fatty acid spectra. Its content in cell lipids was over 70%, which corresponds to the literature data on the fatty acid spectrum of the genus *Comamonas* bacteria (**Tamaoka *et al.*, 1987**). The content of marker hydroxy acids (2 hydroxyhexadecanoic (C16: 0 2OH) and 3-hydroxydecanoic (C10: 0 3OH)) for typical species of the genera *Comamonas* spp and *Delftia* spp in the cell lipids of the studied isolates # 46 and # 47 ranged from 2-5% from the total area of the peaks (**Table 2**), which is consistent with the literature (**Tamaoka *et al*, 1987; Wen, 1999**). In the fatty acid spectrum of the isolates, hexadecanoic acid (# 46 - 29.56 ± 0.48%; # 47 - 30.28 ± 0.64%), hexadecenoic acid (# 46 - 30.77 ± 1.0; # 47 - 32.78 ± 0.3), octadecenoic (# 46 - 24.54 ± 0.45%; # 47 - 23.5 ± 1.09%) were presented in the predominant amount, as well as minor acids: 3-hydroxydecanoic in the amount by 2.57 ± 0.32% and 2.33 ± 0.02%, respectively, and 2-hydroxyhexadecanoic (2.56 ± 0.34% and 1.83 ± 0.05%, respectively).

Table 2 Fatty acid composition of cellular lipids of reference and test strains (% of total content)

	Comamonas testosteroni UCM B-213	*Delftia acidovorans* UCM B-197	*Comamonas terrigena* UCM B-363	*Pseudomonas putida* UCM B-115	Isolate #46	Isolate #47
$C_{10:0\ 3OH}$	3.82 ± 0.25	2.36 ± 0.20	4.01 ± 0.49	4.18 ± 0.37	2.57 ± 0.32	2.33 ± 0.02
$C_{12:0\ 2OH}$	-	-	-	4.77 ± 0.2	-	-
$C_{12:0\ 3OH}$	-	-	-	1.95 ± 0.1	-	-
$C_{12:0}$	3.31 ± 0.27	3.23 ± 0.80	3.95 ± 0.31	t	2.93 ± 0.38	2.67 ± 0.33
$C_{14:0}$	0.35 ± 0.05	0.46 ± 0.21	3.17 ± 0.62	0.50 ± 0.03	0.39 ± 0.18	0.46 ± 0.21
$C_{15:0}$	-	-	1.45 ± 0.60	t	t	t
$C_{16:0}$	29.80 ± 1.73	23.73 ± 0.18	34.8 ± 1.01	24.73 ± 1.37	29.56 ± 0.48	30.28 ± 0.64
$C_{16:0\ 2OH}$	2.72 ± 0.58	-	t	t	2.56 ± 0.34	1.83 ± 0.05
$C_{16:\ 1\ cis\ 9}$	30.83 ± 1.81	39.47 ± 0.46	37.49 ± 0.97	17.61 ± 1.27	30.77 ± 1.0	32.78 ± 0.3
$C_{17:0\ cyclo}$	2.40 ± 0.39	t	0.50 ± 0.16	t	2.57 ± 0.26	2.46 ± 0.4
$C_{17:0}$	1.37 ± 0.46	t	0.59 ± 0.12	t	1.28 ± 0.1	1.05 ± 0.05
$C_{18:1\ cis9}$	22.52 ± 0.5	29.98 ± 0.82	12.56 ± 0.85	24.49 ± 1.48	24.54 ± 0.45	23.5 ± 1.09
$C_{18:0}$	0.76	0.26 ± 0.03	t	2.21 ± 0.17	0.43 ± 0.08	1.17 ± 0.03

Legend: «-» – not detected; «t» – trace – less than 0.5%; ± SD (standard deviation)

Analysing fatty acid spectra of cell lipids of studied, as well as *D. acidovorans* UCM B-197, *C.testosteroni* UCM B-213, *C. terrigena* UCM B-363, *P.putida* B-115 collection strains, confirmed the belonging the isolate # 47 to *C. testosteroni*. The affiliate of isolate #46 to the species *D. acidovorans* is questionable due to the presence in the fatty acid spectrum of the 2-hydroxyhexadecanoic acid (C16: 0 2OH), not to be characteristic for the genus *Delftia*, and according to the literature is characteristic of the species *Comamonas testosteroni* cellular lipids. The fatty acid compositing cell lipids of *C. terrigena* B-363 was studied as a negative control, because *C. terrigena* is a typical species from the genus *Comamonas*, and the species *P. putida* UCM B-115 was included in the study as one of the most common species of the genus *Pseudomonas*, which previously included the species *D. acidovorans* and *C. testosterone* (**Palleroni, 1984**). The qualitative and quantitative fatty acids composition of total lipids in *C. testosterone* UCM B-213 and *D. acidovorans* UCM B-197 were similar. The exception was 2-hydroxyhexadecanoic acid, as absented in the cell lipids of *D. acidovorans* UCM B-197. However, the fatty acid composition of *C. terrigena* UCM B-363 was slightly different from *C. testosteroni* UCM B-213. Thus, the contenting pentadecanoic acid was almost 1.5% compared to other strains, 2-hydroxyhexadecanoic was present in small quantity, less than 1%, and tetradecanoic more than 3% by the total composition. Regarding *P. putida* UCM B-115, the fatty acid composition also contained two markers for pseudomonads hydroxy acids - 2-hydroxydodecanoic and 3-hydroxydodecanoic acids, its contents were less than 5% of the total composition. Comparing the qualitative and quantitative fatty acids composition of reference strains as *C. testosteroni* UCM B-213 and *D. acidovorans* UCM B-197, the fatty acid compositions of isolates # 46 and # 47 were concluded to be similarity to *C. testosteroni* UCM B-213 and be differ to *C. terrigena* UCM B-363 and *P. putida* B-115.

According to the literature, the fatty acid composition of *Comamonas* cellular lipids is characterized by the presence of three major (quantitative content is more than 70%) acids: hexadecanoic, hexadecenoic, octadecenoic, and one minor - 3-hydroxydecanoic acid. An important indicator for representatives of this genus is the presence of 2-hydroxyhexadecanoic acid, but in different amounts. For example, in a typical representative *C. terrigena*, its content is less than 1%, in *C. testosteroni* - from 2 to 7%, in *C. koreensis* - less than 4% (**Chang, 2002**). In contrast, marker acids for the representatives of the genus *Pseudomonas*, beside the above are also 2-hydroxydodecanoic and 3-hydroxydodecanoic acids, which was one of the reasons for the reclassification of *P. acidovorans* species to *C.acidovorans*, and subsequently - to *D. acidovorans*. *P. testosteroni* was also reclassified to *C. testosteroni*. In addition, many species of the genus *Pseudomonas* were subsequently reclassified to new genera *Acidovorax*, *Variovorax*, *Hydrogenophaga* of the family *Comamonadaceae* (**Ikemoto, 1978; Tamaoka, 1987; Stead, 1992; Wen, 1999**). The 2-hydroxyhexadecanoic acid presence in the cell lipids distinguishes representatives of *C. testosteroni* species from *D. acidovorans*, in the fatty acid spectra of which it is absent. Only one study indicated that in some *D.acidovorans* strains the 2-hydroxyhexadecanoic acid amount was less than 1%, namely this is only a strain feature not to apply as whole to most members. This can be confirmed by a more recent polyphasic taxonomic analysis of strain *D. acidovorans* ESM-1 (**Ashraf et al. 2019**), where 2-hydroxyhexadecanoic acid in the fatty acid composition was not detected. In addition to marker acids commoning to the family *Comamonadaceae*, bacteria of the genera *Delftia* and *Acidovorax* also have 3-hydroxyoctanoic acid, distinguished its from the representatives of the genus *Comamonas* (**Gardan, 2003**).

Genomic characterization

According to the resulting the 16S rRNA gene sequencing of isolates # 46 and # 47, sequencing of this genes were obtained with sizes: 1437 np and 1487 np, respectively, which was deposited in GenBank under the following numbers: SUB9376895 Comamonas_testosteroni_46 MW861636, SUB9376895 Comamonas_testosteroni_47 MW861637.
Analysying the affinity of the 16S rRNA gene nucleotide sequences of isolates # 46 and # 47 with the deposited sequences of families *Pseudomonadaceae, Burkholderiaceae, Comamonadaceae* found that they are most closely related to the family *Comamonadaceae* (Fig. 1).

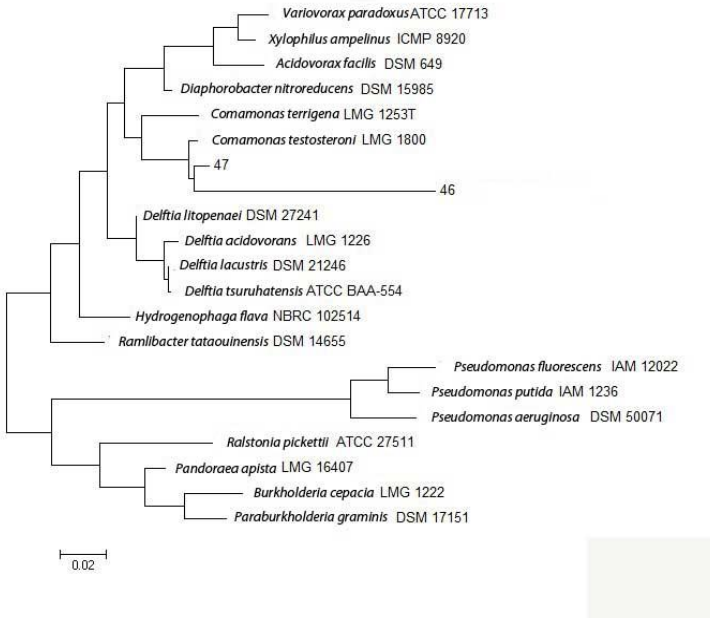

Figure 1 The affinitying the 16S rRNA gene nucleotide sequences of isolates # 46 and # 47 and the 16S rRNA gene nucleotide sequences of typical members from the families *Comamonadaceae, Burkholdereaceae and Pseudomonadaceae* stored in the GenBank rRNA database (dendogram was constructed by software Mega 5.0.)

Given the phylogenetic characteristics based on the 16S rRNA gene sequence and the phenotypic properties of isolates # 46 and # 47, it should be noted that on the dendrogram *C.thiooxidans* is in the same cluster with *C. testosteroni* (Fig. 2). *C. thiooxidans* is characterized by the ability to assimilate sugars: glucose, lactose, galactose, some amino acids, as well as to produce H2S, not be characteristic for *C. testosteroni*. Regarding chemotaxonomic features, the comparative characteristics of these two species as showing *C.thiooxidans* to differ from *C. testosteroni* by the following fatty acids composition: heptadecenoic (C17: 1 x8c), 2-hydroxytetradecanoic (C14:0 2OH), 11-methyl-octadecenoic (C11 Methyl 18: 1 x7c) and cyclononadecanoic (C19:0 cyclo x8c) (**Kunwar et al., 2010**).

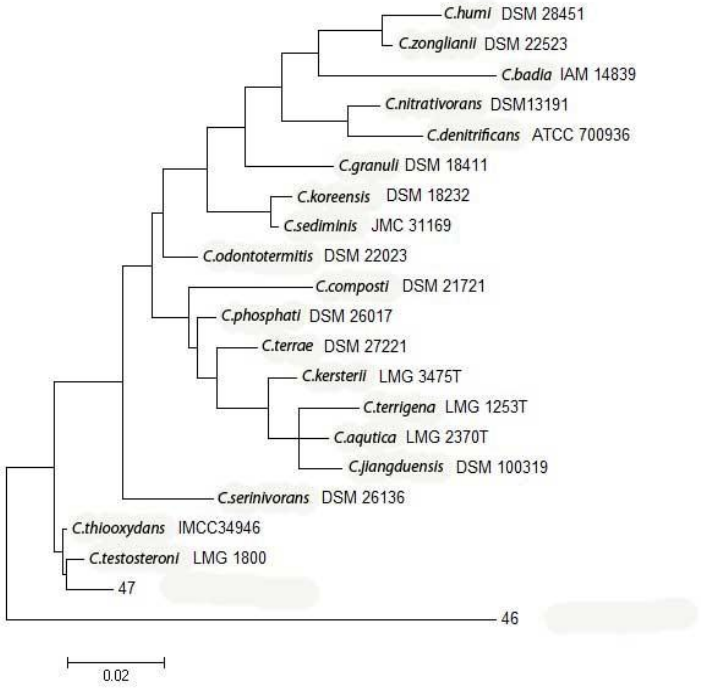

Figure 2 The affinity of the nucleotide sequences of the 16Sr RNA gene of isolates # 46 and # 47 and the nucleotide sequences of the 16S rRNA gene of the genus Comamonas from the GenBank database

According to phylogenetic analysis, isolate # 47 was 98.05% related to *C. testosteroni* LMG 1800, and according to biochemical properties by 99% with strain of *C. testosteroni* UCM B-213. The 16S rRNA gene nucleotide sequence analysying the isolate # 46 with a typical strain of *D. acidovorans* LMG 1226 revealed affinitying only by 95.25%, as casted to doubt on the affiliating this strain to the species *D. acidovorans*, but phylogenetically, strain # 46 was related to *C. testosteroni* LMG 1800 by 97.77%, giving grounds to attribute it to the species *C. testosteroni*.
Therefore, investigation have shown that the studied strains # 46 and # 47 belong to the species Comamonas testosteroni, Comamonas genus, Comamonadaceae family, Burkholderiales order, Betaproteobacteria class, Proteobacteria phylum, Bacteria kingdom. They are characterized by the following phenotypic features: gram-negative, aerobic, motile, non-spore-forming rods, which are distributed in various ecotopes, including soil. The optimum growth temperature is +30 ºC. Various organic acids and amino acids are used as a source of carbon, but most carbohydrates are not utilized, even glucose, also strains are not capable of denitrification, do not produce hydrogen sulfide.
The representatives of the genus Comamonas are known to be used in environmental technologies - the pollutant destructions for bioremediation of contaminated areas. In a continuously operating biofilm reactor with a C. testosteroni's fixed layer was used to degrade 4 toluenesulfonic acid. The strain C. terrigena N3H was able to decompose the anionic surfactant dihexyl sulfosuccinate. In immobilized cell reactors, m- and p-nitrobenzoate were destroyed using Comamonas JS46 and JS47. There are known destruction pathways of organochlorine compounds by Comamonas sp.: chloronitrobenzene, 4-chloronitrobenzene (4CNB) (**Liu, 2007; Wu, 2006; Katsivela, 1999**). The ability of Comamonas sp to use 4CNB as the sole sourcing carbon and energy has been reported (**Zhang, 2009**). Thus, the genus Comamonas members are promising to study its ability to destroy environmentally hazardous pollutants, including organochlorine compounds.

CONCLUSION

Polyphase taxonomic analysis was used to determine the taxonomic position of soil bacterial isolates found from the organochlorine waste landfill from the chemical enterprice in Kalush, Ivano-Frankivsk region of Ukraine. Bacterial isolates # 46 and # 47 showed resistance to high HCB concentration (50 $mg*l^{-1}$). The isolates are motile gram-negative rods, aerobic, chemoorganotrophic. According to chemotaxonomic features they were characterized by the ability to assimilate lactate, malate, elman, succinate and lactate alkalis, showed glutamylarylamidase, pyrrolidone-arylamidase, tyrosinarylamidase, gamma glutamyltransferase activities, not assimilate glucose, lactose, trehalose and other sugars, not utilize malonate, sodium citrate, not produce hydrogen sulfide. In the fatty acid composition of cellular lipids fatty acids with a carbon chain length from C10 to C18, saturated, unsaturated and hydroxy acids were found. The contenting markers hydroxy acids for the typical genus Comamonas: 2 hydroxyhexadecanoic

(C16: 0 2OH) and 3 hydroxydecane (C10: 0 3OH) in cellular lipids ranged from 2-5%, corresponded to its content in members of this genus. According to the16S rRNA gene sequencing, the affinity the nucleotide sequences of isolates # 46 (97.77%) and # 47 (98.05%) with a typical Comamonas testosteroni LMG 1800 strain from GenBank database was determined. Based on the obtained data, the studied isolates were assigned to Comamonas testosteroni, deposited in GenBank under the numbers SUB9376895 Comamonas_testosteroni_46 MW861636, SUB9376895 Comamonas_testosteroni_47 MW861637 and included in the Ukrainian Collection of Microorganisms under the numbers UCM B-400 and UCM B-401, respectively.

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