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ENVIRONMENTAL BIOTECHNOLOGY

Diversity and degradative capabilities of bacteria and fungi isolated from oil-contaminated and hydrocarbon-polluted soils in Kazakhstan

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Abstract

Bacteria and fungi were isolated from eight different soil samples from different regions in Kazakhstan contaminated with oil or salt or aromatic compounds. For the isolation of the organisms, we used, on the one hand, typical hydrocarbons such as the well utilizable aliphatic alkane tetradecane, the hardly degradable multiple-branched alkane pristane, and the biaromatic compound biphenyl as enrichment substrates. On the other hand, we also used oxygenated derivatives of alicyclic and monoaromatic hydrocarbons, such as cyclohexanone and p-*tert*-amylphenol, which are known as problematic pollutants. Seventy-nine bacterial and fungal strains were isolated, and 32 of them that were clearly able to metabolize some of these substrates, as tested by HPLC-UV/Vis and GC-MS analyses, were characterized taxonomically by DNA sequencing. Sixty-two percent of the 32 isolated strains from 14 different genera belong to well-described hydrocarbon degraders like some Rhodococci as well as *Acinetobacter*, *Pseudomonas*, *Fusarium*, *Candida*, and *Yarrowia* species. However, species of the bacterial genus *Curtobacterium*, the yeast genera *Lodderomyces* and *Pseudozyma*, as well as the filamentous fungal genera *Purpureocillium* and *Sarocladium*, which have rarely been described as hydrocarbon degrading, were isolated and shown to be efficient tetradecane degraders, mostly via monoterminal oxidation. Pristane was exclusively degraded by *Rhodococcus* isolates. *Candida parapsilosis*, *Fusarium oxysporum*, *Fusarium solani*, and *Rhodotorula mucilaginosa* degraded cyclohexanone, and in doing so accumulate ε-caprolactone or hexanedioic acid as metabolites. Biphenyl was transformed by *Pseudomonas/Stenotrophomonas* isolates. When p-*tert*-amylphenol was used as growth substrate, none of the isolated strains were able to use it.

Keywords Degradation · Crude oil · *n*-Alkanes · Cycloalkanes · Cyclohexane · *Bordetella* · *Paecilomyces*

Introduction

Kazakhstan is one of the fifteens most important oil-producing countries worldwide with around 1956 thousand barrel a day (Trading Economics 2019) and it is the twelfth largest oil-exporting nation with 68.1 million tons a year (Statista 2019)—a value that is still rising. Oil production and oil transport are frequently accompanied by spills that pollute the environment. To respond in an appropriate way, much effort worldwide has been—and is still being—invested in remediation, particularly in bioremediation (Bento et al. 2005; Juwarkar et al. 2010; Varjani et al. 2017). Among the various options, biodegradation with microorganisms seems to be one of the most used and cost-effective procedures for remediation of oil pollution. In Kazakhstan, we isolated various highly potent microbial oil degraders from contaminated soils of the Uzen deposit (Mangystau region) and of the Kumkol deposit (Kyzylorda region). These isolates utilized a large number of oil components and by degrading pollutants had beneficial effects on the growth of barley seeds in the presence of crude oil (Mikolasch et al. 2015, 2016). The question has also been raised whether microorganisms of the same quality and quantity can be isolated from areas with different oil pollutants and whether correlations concerning biodiversity and metabolic activity can be drawn. Furthermore, very

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little is known about the diversity of species and the degradation or transformation potential of microorganisms from other than heavily oil-polluted deposits in Kazakhstan. For this reason, samples from different contaminated soils were taken during an expedition from the north bank of the Lake Balkhash to the Talgar Pass (Tien Shan Mountains). These samples were characterized as to their biodiversity and metabolic activity. So that suitable strains would be available for further biotechnological and biochemical purposes, characterization was carried out not by analysis of the microbiome by extraction of rRNA/DNA, but rather by isolation and analysis of highly effective degraders in culture. For this reason, samples were grown on mineral media containing either typical hydrocarbons such as the well utilizable aliphatic alkane tetradecane, the hardly utilizable multiple-branched alkane pristane, and the biaromatic compound biphenyl or the oxygenated derivatives of alicyclic and monoaromatic hydrocarbons such as cyclohexanone and p-*tert*-amylphenol, which are known to be problematic pollutants. Many microorganisms can oxidize cyclohexane to cyclohexanone, but further metabolism of the oxygenated alicyclic cyclohexanone is difficult for many organisms (Dallinger et al. 2016). Furthermore, the branched side chain of p-*tert*-amylphenol prevents a ready further oxidation of this phenol derivative. An additional aim of this study was therefore to identify effective new degraders for pollutants that are otherwise difficult to degrade.

Materials and methods

Sampling

Soil samples were collected at eight different locations in Kazakhstan (samples E1–E8 in Table 1) in order to have different samples of material contaminated with oil or salt or aromatics.

Media

Nutrient broth II (SIFIN, Berlin) (NB) and malt agar (2.5% malt extract) (MA) served as cultivation media on plates.

Mineral salts medium for bacteria (MSMB) pH 6.3 (Hundt et al. 1998) and mineral salts medium for fungi (MSMF) pH 5.4 supplemented with 1% vitamin solution (Awe et al. 2008) were used for the isolation of microorganisms from various polluted soils and, without agar, in liquid form for metabolic experiments.

Enrichment and isolation

Bacteria and fungi from the contaminated soil samples were enriched on the substrates tetradecane, pristane, cyclohexanone, biphenyl, and 4-*tert*-amylphenol (Supplementary Material Table S1) each with MSMB and MSMF according to a previously described method (Nhi-Cong et al. 2010).

After enrichment, fungi were obtained by plating 0.1 mL of the enriched cultures in MSMF on MA plates. Bacteria enriched in MSMB were plated on NB plates. Pure cultures from MSMB were cultivated on NB and from MSMF on MA slants.

Evaluation of growth on isolation substrates

For growth tests in liquid medium, microorganisms were cultivated in 500-mL flasks containing 100 mL of MSMB or MSMF supplemented with the isolation substrates (see concentrations in Supplementary Material Table S1) at 30 °C and 130 rpm, harvested, dried, and analyzed. Strains, which showed satisfactory growth after 6 days, were then used for further treatment. Cultures with slower growth on the substrates were harvested after 12 days. The experiments with the substrate cyclohexanone were carried out with glass in place of cellulose stoppers, due to the excessive evaporation of this substrate.

Because of difficulties in quantification of cell densities by optical density (OD) measurements, we analyzed cell growth by the dry weight method according to Mikolasch et al. (2015). For this, the final cell weight minus the cell weight at the beginning was divided by the cell weight at the beginning. The growth values are therefore dimensionless.

$$\text{Growth value} = (\text{cell weight end [mg]} - \text{cell weight start [mg]})\,(\text{cell weight start [mg]})^{-1}$$

Identification of isolated microorganisms

Isolates were first identified as bacteria, yeasts, or fungi using a phase-contrast microscope (Axiolab, Zeiss). Initial assignments were made on the basis of shape, mobility, formation of endospores, pullulation, and colony morphology of the isolates.

Identification of bacteria

The KOH test was used to determine the Gram characteristics of all bacteria (Suslow et al. 1982). To distinguish between aerobic and facultative anaerobic bacteria, we used the oxidative/fermentative test according to Hugh and Leifson (1953). Furthermore, all bacteria were grown on plates for 48 h and chromosomal DNA was isolated in triplicate by using cell material of one colony in 20 μL ddH_2O and the maximal power of a microwave (Severin, Germany) for 3 min. After centrifugation for 1 min at 12,000 rpm, 1 μL supernatant was used as template in PCR. This isolation method is referred to as *method 1*. For the cells of strains that could not be disrupted by this method, we used the innuSPEED soil DNA-Kit (Analytic Jena AG, Germany) according to the manufacturer's

Table 1 Sampling sites from eight different polluted locations in Kazakhstan

Sample	Amount [g]	Region	Isolation place	Latitude/ longitude
E1	1.2	Shyghanag	Petrol station (still operating)	+ 45.1°/+ 73.7°
E2	1	Lake Balkhash, close by Ortaderesin	Salty soil bankside	+ 46.74°/+ 75.5°
E3	2	Ülken	Petrol station (out of service)	+ 45.3°/+ 73.8°
E4	1.6	Agsügek	Petrol station (out of service)	+ 44.6°/+ 74.6°
E5	1.4	Quaskeleng	Petrol station (out of service)	+ 43.3°/+ 76.6°
E6	32	Quasattung	Petrol station (out of service)	+ 43.6°/+ 78.0°
E7	32	Shilik	Petrol station (out of service)	+ 43.6°/+ 78.3°
E8	16	Talgar Pass/Tian Shan Mountains	Terminal cableway station under construction (3162 m)	+ 43.1°/+ 77.1°

protocol. This method is referred to as *method 2*. For strains whose cells could not be disrupted by either of these methods, we disrupted the cells using a Fastprep24 instrument (MP Biomedicals, Germany) in combination with the DNeasy PowerSoil Kit (Qiagen, US)—referred to here as *method 3*.

Bacterial almost full-length 16S rRNA genes were amplified from 1 μL DNA extracts as template with the oligonucleotide primers 616V (AGAGTTTGATYMTGGCTC, 0.5 μM) (Juretschko et al. 1998) and 1492R (GGTTACCTTGTTAC GACTT, 0.5 μM) (Kane et al. 1993) and with $MgCl_2$ 2 mM, dNTPs mix 0.2 mM, and BSA 0.2 mg mL^{-1} (Eurofins Genomics, Germany). Reactions were performed in an Analytik Jena thermocycler using GoTaq-Polymerase (Eurofins Genomics, Germany) with the following conditions: step 1 denaturation 95 °C 5 min, step 2 denaturation 95 °C 30 s, step 3 annealing 47 °C 45 s, step 4 elongation 72 °C 90 s. Steps 2 to 4 were repeated 34 times, followed by elongation at 72 °C for 5 min. The resulting PCR products were purified using the DNA Clean & Concentrator Kit D4003 (Zymo Research, Germany). DNA concentrations were determined by nanodrop spectrophotometer (PEQLAB Biotechnologie GmbH). Sanger sequencing was performed by Eurofins Genomics (Germany) with 616V and 1492R primers, respectively. The resulting forward and reverse sequences were assembled using the program Geneious (geneious, US). The resulting almost full-length 16S rRNA sequences were compared with the NCBI nr database using the blastn algorithm (Altschul et al. 1990).

Identification of yeasts and filamentous fungi

The urease test with Christensen's medium (Seeliger 1956) was used to determine ascomycetous and basidiomycetous yeasts. All yeasts were grown for 48 h and chromosomal DNA was isolated by using methods 1, 2, and/or 3 as described above for bacteria. Methods 2 and 3 were used for filamentous fungi.

Fungal almost full-length ITS genes were amplified using 1 μL DNA extract as template with oligonucleotides ITS1 (TCCGTAGGTGAACCTGCGG, 0.5 μM) and ITS4 (TCCTCCGCTTATTGATATGC, 0.5 μM) (White et al. 1990) as primers and with $MgCl_2$ 2 mM, dNTPs mix 0.2 mM, and BSA 0.2 mg mL^{-1} (Eurofins Genomics, Germany). Reactions were performed in an Analytik Jena thermocycler using GoTaq-Polymerase (Eurofins Genomics, Germany) with the following conditions: step 1 denaturation 95 °C 10 min, step 2 denaturation 95 °C 60 s, step 3 annealing 55 °C 60 s, step 4 elongation 72 °C 90 s. Steps 2 to step 4 were repeated 29 times, followed by elongation at 72 °C for 10 min. The resulting PCR products were purified, sent for Sanger sequencing, and analyzed as described above for bacteria.

Metabolic experiments

Pre-cultivated cells of the isolated strains were shaken in 100-mL Erlenmeyer flasks containing 10 mL medium plus the isolation substrates as carbon and energy source at 30 °C and 130 rpm. Assays without substrates and without cells were used as controls.

Bacterial cells were pre-grown on NB plates, for yeasts and filamentous fungi on MA plates. For each strain, the cell material from five well-grown plates was used to prepare an inoculation suspension of 6 mL. In each case, 2 mL of this was used to inoculate the parallel transformation assays and the control flasks. For degradation experiments, fungi were cultivated in MSMF supplemented with 1% vitamin solution and bacteria in MSMB. As sole source of carbon and energy, 25 μL of the isolation substrates tetradecane, pristane, and cyclohexanone was used in 10 mL assays (substrate concentration 0.25% *v*/*v*). Biphenyl was used as substrate according to the method described by Sietmann et al. (2000) at a final concentration of 100 μg mL^{-1}.

After 5 (for bacteria and yeasts) and 7 (for filamentous fungi) days of incubation, the supernatant of each transformation assay was extracted according to Mikolasch et al. (2015). The extracts obtained were analyzed by high-performance liquid chromatography (HPLC) and gas chromatography mass spectrometry (GC-MS). The data are reported as means for

parallel experiments. The deviation of the parallel values was no more than 10%.

Chemical analysis and identification of products

The HPLC-UV/Vis and GC-MS analyses were carried out as described previously (Mikolasch et al. 2016). The elution profile of the GC-MS method was changed as follows: the column temperature started at 80 °C and increased to 310 °C at 10 °C min^{-1} and was finally maintained at 310 °C for 5 min.

Results

To investigate the diversity of species and the degradation or transformation potential of microorganisms, samples from eight different contaminated soils in Kazakhstan were collected during an expedition from the north bank of the Lake Balkhash to the Talgar Pass (Tien Shan Mountains). Five soil samples were taken from the ground of abandoned petrol stations along the route (E3–E7) and one from an old filling station that was still operating (E1). Tetradecane, pristane, and cyclohexanone—all components of crude oil—were used as isolation substrates for samples E1 and E3–E7. One sample was of salty soil from the heavily polluted Lake Balkhash region (E2) (Krupa et al. 2017). The final sample came from the Talgar Pass at a height of 3162 m where there was serious soil pollution with wood preservatives in connection with the construction of wooden cable stations. From this soil sample (E8), we expected microorganisms able to degrade aromatic hydrocarbons; therefore, we used biphenyl and 4-*tert*-amylphenol as isolation substrates. Because of the interest in a biotechnological application of strains effectively degrading pollutants, we concentrated in general on the best-growing microorganisms.

Isolation of and screening for degraders of pollutants

Seventy-nine strains of microorganisms were isolated from 32 enrichment experiments using the five substrates, tetradecane (1), pristane (2), cyclohexanone (3), biphenyl (4), 4-*tert*-amylphenol (5), and the eight soil samples described above (Table 2 and Supplementary Material Table S2).

Thirty-eight cultures enriched on MSMB and 41 strains obtained from MSMF were cultivated in liquid media on the substrates, tetradecane, pristane, cyclohexanone, biphenyl, and 4-*tert*-amylphenol. Due to difficulties in quantification of cell densities via OD measurements, we analyzed the cell growth by a dry weight method (Mikolasch et al. 2015).

Twenty-one of the 37 microorganisms isolated on tetradecane were able to grow on this substrate (Table 3 and Supplementary Material Table S3 and S4). Four of the 21 strains showed good growth values (three from MSMB and one from MSMF), six moderate (one from MSMB and five from MSMF), and 11 weak growth (five from MSMB and six from MSMF). However, more than half of the isolates were able to use tetradecane as the sole source of carbon and energy. The highest growth value 469 (the final cell weight minus the cell weight at the beginning was divided by the cell weight at the beginning) was determined for the strain SBUG 2074, whereas the largest final yield of cell material was measured for SBUG-Y 2192 with 646 mg/100 mL (growth value 134; Supplementary Material Table S2 and S3). In general, the growth values of strains isolated on MSMF were smaller than the ones of strains isolated on MSMB.

The growth on cyclohexanone was much weaker than on tetradecane (Table 3 and Supplementary Material Table S5 and S6). Only six of the 18 strains showed weak growth (one from MSMB and five from MSMF). No strain had better than weak growth values. However, one third of the isolates are able to use cyclohexanone as the sole source of carbon and energy. The highest growth value 37 and the largest final yield of cell material with 159 mg/100 mL were determined for the strain SBUG-M 1735 (Supplementary Material Table S2 and S6). The growth values of the strains isolated on MSMF are similar (three strains) or higher (two strains) than the value of the strain isolated on MSMB.

Three of the nine strains isolated on pristane (all from MSMB) are able to use this substrate as the sole source of carbon and energy (Table 3 and Supplementary Material Table S7 and S8). No strain isolated from MSMF could really grow on the isolation substrate (growth values between four and nine). The strain SBUG 2033 from MSMB showed a moderate growth value of 83 and the largest final yield of cell material with 152 mg/100 mL (Supplementary Material Table S2 and S7).

4-*tert*-Amylphenol was neither used as growth substrate by the six strains isolated in MSMB nor by the seven isolates from MSMF (Supplementary Material Table S9 and S10). The growth values are less than 1.

The two strains isolated on biphenyl showed growth values of 6.6 and 10, which indicates a poor and weak growth (Table 3 and Supplementary Material Table S11).

In order to determine and compare the number of enriched strains with the number of strains which are able to use the isolation substrates as growth substrate, we used the number of isolated pure cultures per soil sample and substrate (Fig. 1a, Supplementary Material Table S12).

By far the highest number of organisms were recovered on tetradecane. This reflects that seven of the eight soil samples were used for enrichment on this substrate whereas only one to three soil samples served as basis for enrichment on the other substrates. However, though two to seven pure cultures were isolated from each enrichment experiment, only one to five strains were able to use the isolation substrate as a sole source of carbon and energy. An exception is 4-*tert*-

Table 2 Number of microorganisms recovered from eight different contaminated locations in Kazakhstan

Substrate	Soil sample	MSMB	MSMF	Sum of isolated strains
Tetradecane	E1	4	3	7
	E2	2	3	5
	E3	2	4	6
	E4	5	2	7
	E5	1	5	6
	E6	1	2	3
	E7	1	2	3
	Sum	16	21	37
Cyclohexanone	E3	3	4	7
	E6	5	1	6
	E7	3	2	5
	Sum	11	7	18
Pristane	E6	3	2	5
	E7	1	3	4
	Sum	4	5	*9*
4-*tert*-Amylphenol	E6	2	3	5
	E7	1	1	2
	E8	3	3	6
	Sum	6	7	13
Biphenyl	E8	1	1	2
Sum of each medium		38	41	
Sum of all microorganisms isolated as pure cultures				79

amylphenol, which could not serve as substrate. Four or five pure cultures were obtained on tetradecane and cyclohexanone, whereas no more than 3 pure strains were isolated on pristane and biphenyl.

Identification of degraders of pollutants

All bacterial isolates able to grow on the isolation substrates were assigned as Gram positive or negative and identified by 16S rRNA sequence analysis, and the oxidative/fermentative test (Hugh and Leifson 1953). The fungi were characterized by ITS gene sequence analyses and the urease test with Christensen's medium (summary in Table 3 and identification details in Supplementary Material Table S13, S14, S15). Bacterial 16S rRNA gene sequences and fungal ITS sequences were deposited in Genbank under the accession numbers listed in Table 3. As is current practice in the literature, we considered only those microorganisms having the desired characteristics (Martorell et al. 2017).

The 32 identified strains belong to 14 different genera. Forty-four percent of the strains are bacteria and 56% are fungi. The bacterial species belong to the *Firmicutes* (21%), *Proteobacteria* (36%; 80% *Gamma*- and 20% *Betaproteobacteria*), and *Actinobacteria* (43%). The fungi are divided into 22% *Basidiomycota* and 78% *Ascomycota*. The percentage of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Ascomycota*, and *Basidiomycota* for the strains able to grow on the isolation substrates can be inferred from the circular charts of Fig. 1b. The highest number of different strains was found with the more easily useable substrate tetradecane followed by cyclohexanone. Only few bacterial strains belonging to the genera *Pseudomonas* and *Rhodococcus* were isolated and able to grow on the more recalcitrant substrates pristane and biphenyl, whereas 4-*tert*-amylphenol was not a growth substrate.

Verification of the degradation potentials by analyses of intermediates

To confirm that the identified pollutant degraders have a high metabolic activity toward the isolation substrates, transformation experiments in liquid medium were conducted on the isolation substances, and the metabolites were detected by GC-MS or HPLC (Table 4).

Most of the tetradecane degraders were able to transform 50% or more of the substrate, six of the 21 transforming 100% of it. Two or more monocarboxylic acids, mostly myristic (1-tetradecanoic) and lauric (1-dodecanoic) acid, were detected in each assay. Metabolism of strains utilizing tetradecane was confirmed by their formation of monocarboxylic acids. In contrast, a dicarboxylic acid was formed only by the two species, *Purpureocillium lilacinus* SBUG-M 1730 and *Yarrowia*

Table 3 Identified microorganisms from eight different contaminated locations in Kazakhstan

SBUG number	Genbank accession number	Growth value [a)]	Best hit(s) of gene sequence analysis	Ident [%] 16S rRNA	Ident [%] ITS	Sample site
Isolation substrate **tetradecane**						
Isolation medium MSMB						
2074	MH043938	469	*Bacillus licheniformis*	100		E7
2092	MH043940	36	*Bacillus subtilis*	100		E1
2090	MH043939	13	*Bacillus* sp.	98		E1
2083	MH043941	69	*Bordetella* sp.	99		E4
2075	MH043949	27	*Rhodococcus* sp.	100		E3
2093	MH043950	14	*Rhodococcus* sp.	99		E4
-M 1730	MH059649	139	*Purpureocillium lilacinus*[b)]		96	E4
-Y 2201	MH059656	10	*Rhodotorula mucilaginosa*		99	E4
-Y 2192	MH059658	134	*Yarrowia lipolytica*		97	E6
Isolation medium MSMF						
2094	MH043942	16	*Curtobacterium* sp.	99		E5
2097	MH043943	14	*Curtobacterium* sp.	99		E5
-M 1727	MH059642	79	*Fusarium oxysporum*		99	E1
-M 1731	MH059645	70	*Fusarium* sp.		96	E5
-M 1732	MH059646	56	*Fusarium sp.*		96	E7
-M 1729	MH059648	105	*Purpureocillium lilacinus*		97	E4
-M 1728	MH059650	66	*Sarocladium* sp.		100	E3
-Y 2196	MH059652	13	*Lodderomyces elongisporus*		99	E2
-Y 2194	MH059654	18	*Pseudozyma aphidis*		99	E6
-Y 2195	MH059653	17	*Pseudozyma aphidis*		99	E7
-Y 2193	MH059655	16	*Pseudozyma* sp. or *Sporisorium* sp.		99	E5
-Y 2204	MH059659	63	*Yarrowia lipolytica*		96	E6
Isolation substrate **cyclohexanone**						
Isolation medium MSMB						
2088	MH043937	15	*Acinetobacter* sp.	99		E6
Isolation medium MSMF						
-M 1733	MH059643	26	*Fusarium oxysporum*		99	E6
-M 1735	MH059644	37	*Fusarium solani*		100	E7
-M 1734	MH059647	16	*Fusarium* sp.		94	E7
-Y 2203	MH059651	10	*Candida parapsilosis*		98	E3
-Y 2202	MH059657	14	*Rhodotorula mucilaginosa*		99	E3
Isolation substrate **pristane**						
Isolation medium MSMB						
2096	MH043945	10	*Pseudomonas* sp. / *Stenotrophomonas* sp.	99		E6
2031	MH043947	17	*Rhodococcus* sp.	99		E6
2033	MH043948	83	*Rhodococcus* sp.	100		E6
Isolation substrate **biphenyl**						
Isolation medium MSMB						
2091	MH043944	6.6	*Pseudomonas* sp. / *Stenotrophomonas* sp.	99		E8
Isolation medium MSMF						
2067	MH043946	10	*Pseudomonas* sp.	99		E8

[a)] Growth value > 100 good growth (black), growth value 50–100 moderate growth (dark gray), growth value 10–50 weak growth (light gray), growth value < 10 poor or no growth (white)

[b)] Very light gray underlined species names indicate that either yeasts or filamentous fungi were isolated from MSMB or bacteria were isolated from MSMF

lipolytica SBUG-Y 2204. Three other species, *Fusarium oxysporum* SBUG-M 1727, *Fusarium* sp. SBUG-M, and *Sarocladium* sp. SBUG-M1728, also formed ketones during the transformation of tetradecane.

Two of the cyclohexanone degraders were able to metabolize more than 50% of the substrate, another two metabolized more than 40%, and the remaining three metabolized less than 20%. Metabolites were detected from five of the seven species.

Two of the three pristane degraders utilized more than 80% of the substrate, and *Rhodococcus* sp. SBUG 2031 metabolized more than 95% of it. In both cases, monomethyl substituted alkanedioic and alkenedioic acids were produced.

More than 50% of the aromatic substrate biphenyl was transformed by *Pseudomonas*/*Stenotrophomonas* SBUG 2091 and *Pseudomonas* sp. SBUG 2067. Phenyl-succinic acid and hydroxylated benzoic acids were detected as metabolites by HPLC.

Discussion

From the eight samples of different contaminated soils collected between the north bank of the Lake Balkhash to the Talgar Pass in the Tien Shan Mountains, we isolated 32 oil component degraders belonging to the bacterial phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria* and to the fungal divisions *Basidiomycota* and *Ascomycota*. Fifteen percent of these strains, two *Curtobacterium*, two *Purpureocillium*, and one *Sarocladium*, have not been extensively described in literature, and isolated pure cultures have not so far been shown to be hydrocarbon degraders, though *Curtobacterium* and *Sarocladium* have occasionally been described in oil-contaminated soils (Mohammadian et al. 2017; Rajaei et al. 2013). It is now clear that they are powerful tetradecane degraders (Table 4).

The genus *Curtobacterium* has been defined for several brevibacteria and some coryneform bacteria (Collins and

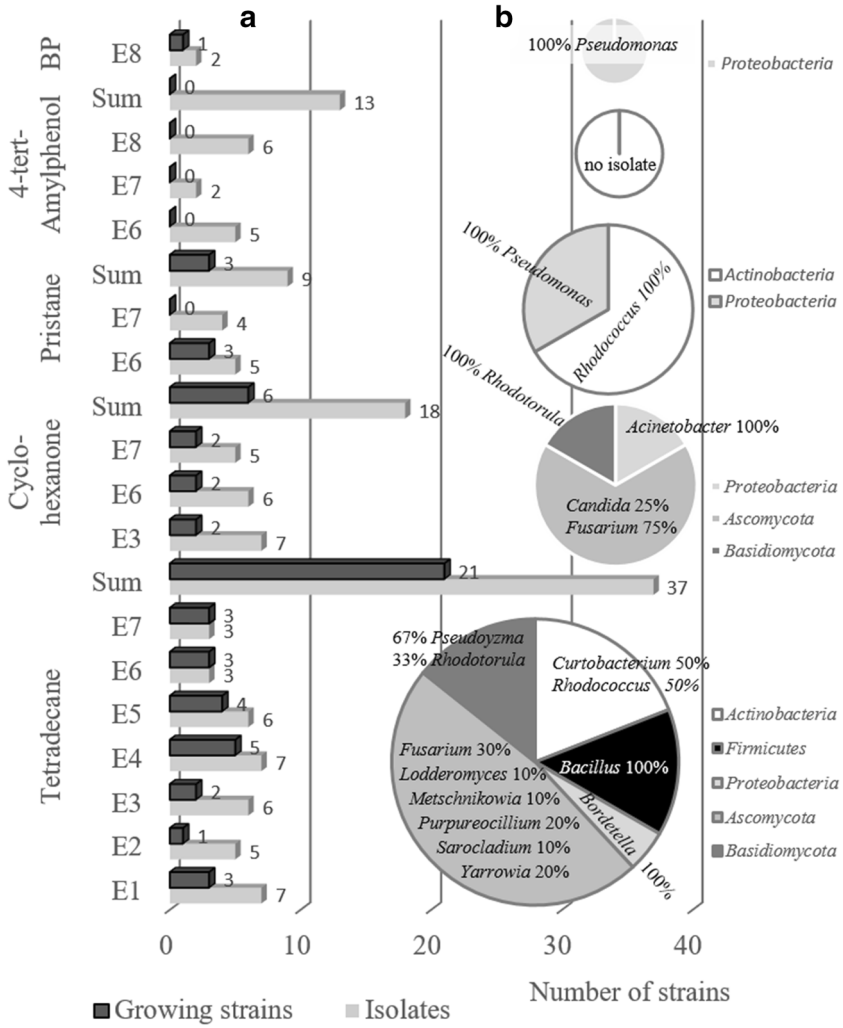

Fig. 1 (**a**, bar chart) Number of isolated microorganisms and number of strains able to grow on the isolation substrate per soil sample and substrate, and the sum of strains per substrate. (**b**, circular charts) Percentage distribution of phyla and genera. The diversity of genera within the phyla is illustrated by genus names and percent values. Each circular chart represents the strains able to grow on the corresponding isolation substrate. (No strain was able to grow on 4-*tert*-amylphenol)

Jones 1983; Funke et al. 2005; Yamada and Komagata 1972). Although the utilization of *n*-alkanes was described in the past for several undefined *Corynebacterium* species (Bouchez-Naitali et al. 1999; Cardini and Jurtshuk 1968; Ikeda et al. 1994; McGowan et al. 2004), the integration of these bacteria into the genus *Curtobacterium* remains unclear. Chase et al. (2016) concluded that *Curtobacterium* may be a cosmopolitan and dominant player in the functional breakdown of dead organic material in leaf litter microbial communities (with high representation on grasses and in soils), and has the ability to utilize different carbohydrates such as starch, cellulose, or xylan. However, there are various different hydrocarbons in the cuticula of plant leaves, so that our characterization of *Curtobacterium* species as hydrocarbon degraders may not be surprising.

Fungi of the genus *Sarocladium* include nearly 20 species belonging to the *Ascomycota* order *Hypocreales*. They have been reported as pathogenic plant fungi or as saprobes, and many of them have been transferred from the morphologically similar (but phylogenetically rather distant) genus *Acremonium* to *Sarocladium* (Giraldo et al. 2015; Summerbell et al. 2011). While *Sarocladium* species are not known as alkane degraders, several *Acremonium* species have been described as hydrocarbon-assimilating (Barnes et al. 2018; Chaineau et al. 1999; Ma et al. 2015; Oudot et al. 1993).

Purpureocillium lilacinus is a ubiquitous saprobic filamentous fungus distributed in soil, decaying vegetation. It is a cause of infection in nematodes, insects, and humans, and belongs also to the *Ascomycota* order *Hypocreales*. Alkane degradation has not been described for the species *Purpureocillium lilacinus* (Luangsa-Ard et al. 2011), which was previously known as *Paecilomyces lilacinus*. For *Paecilomyces lilacinus*, there are several reports concerning the degradation of aromatic compounds such as toluene (Vigueras et al. 2008), benzo(a)pyrene (Rafin et al. 2013), biphenyl (Gesell et al. 2001; Sietmann et al. 2006), or dibenzofuran (Gesell et al. 2004). To date, there is a single report concerning hexadecane utilization by *Paecilomyces lilacinus* (Vigueras et al. 2014). Apart from *Paecilomyces lilacinus*, some other *Paecilomyces* species such as *P. variotii* or *P. brunneolus* remained in the genus *Paecilomyces*. *P. variotii* was described as a petroleum hydrocarbon degrader (Ameen et al. 2016; Ezekoye et al. 2018). In view of this, our characterization of *Purpureocillium lilacinus* as a hydrocarbon degrader is not so surprising.

Our isolates of *Curtobacterium*, *Sarocladium*, and *Purpureocillium* degraded from 70 to 100% of tetradecane and produced several monocarboxylic acids using this substrate. This indicates a monoterminal alkane degradation by initial hydroxylation of tetradecane, oxidation to tetradecanoic acid and its further metabolism by ß-oxidation (Rojo 2009). The detection of 6-tetradecanone in the culture medium of *Sarocladium* sp. SBUG-M1728 indicated the possibility of an additional subterminal degradation pathway for this strain, and the identification of hexanedioic acid generated by *Purpureocillium lilacinus* SBUG-M 1730 indicated a diterminal oxidation pathway (Okuhara et al. 1971; Scheller et al. 1998).

Little is known about hydrocarbon degradation for the genera *Bordetella*, *Lodderomyces*, *Pseudozyma*, and *Rhodotorula*. Species of the bacterial genus *Bordetella* have been isolated as respiratory pathogens in humans and animals but they are frequently found as inhabitants of soils, water, sediments, or the plant rhizosphere (Soumana et al. 2017). Some of them (e.g., *B. petrii* and *B. avium*) have been suggested to be able to degrade benzenes (Wang et al. 2007) and PAHs (Abo-State et al. 2018; Obi et al. 2016; Yuan et al. 2009) whereas our isolate *Bordetella* sp. SBUG 2083 (similar to the cluster of *Bordetella bronchialis/flabilis*; Supplementary Material Table S13) is a tetradecane degrader. Several taxonomic analyses indicated a great similarity between the genera *Bordetella*, *Achromobacter*, and some *Alcaligenes* species partially reclassified to *Achromobacter* (Kronvall et al. 2000; Yabuuchi et al. 1998). For several *Achromobacter* species, the utilization of *n*-alkanes has been described (Deng et al. 2014; Kaczorek et al. 2013; Lal and Khanna 1996).

Our yeast isolate *Lodderomyces elongisporus* SBUG-Y 2196 is a moderate transformer of tetradecane, from which it produced monocarboxylic acids. *L. elongisporus* is known as a hexadecane utilizing yeast species (Bos and de Bruyn 1973; Hofmann and Schauer 1988; Kurtzman et al. 2011), but metabolic studies have not been performed until now.

Whereas other species of the genus *Pseudozyma* (*P. antarctica* and *P.* sp.) are known to metabolize *n*-alkanes (Kitamoto et al. 2001; Sajna et al. 2015), this has not previously been reported for *Pseudozyma aphidis* (Kurtzman et al. 2011). Here, we demonstrate that *Pseudozyma aphidis* SBUG-Y 2194 is able to almost completely degrade tetradecane and to generate thereby four monocarboxylic acids.

Rhodotorula mucilaginosa has previously been described as a degrader of aromatic hydrocarbons (Chandran and Das 2012; Lahav et al. 2002; Romero et al. 2002), whereas our isolate, *Rhodotorula mucilaginosa* SBUG-Y 2201, is a powerful *n*-alkane degrader. Several other *Rhodotorula* species are known as alkane degrading (Kurtzman et al. 2011; Schauer and Schauer 1986), but for *R. mucilaginosa*, the results are contradictory (Hofmann and Schauer 1988). In addition, our isolate of *Rhodotorula mucilaginosa* SBUG-Y 2202 metabolized cyclohexanone to a moderate extent. Current knowledge about fungal degradation of alicyclic hydrocarbons is scarce (Dallinger et al. 2016). Our isolate of *Rhodotorula mucilaginosa* SBUG-Y 2202 transformed cyclohexanone via the previously described microbial pathway to ε-caprolactone and hexanedioic acid (Anderson et al. 1980). The species *Rhodotorula mucilaginosa*, which has so far

Table 4 Transformation experiments on isolation substrates with pure cultures after cultivation for 5 or 7 days

Strain	Amount of remaining substrate [%]	Number of detected products[a)]		
	Tetradecane [%]	Monocarboxylic acids[b)]	Dicarboxylic acids[c)]	Ketones[d)]
Bacillus licheniformis SBUG 2074	82.5 ± 4.5	(2)[e)]	–	–
Bacillus subtilis SBUG 2092	29.1 ± 7.2	2	–	–
Bacillus sp. SBUG 2090	45.7 ± 5.4	(2)	–	–
Bordetella sp. SBUG 2083	84.1 ± 0.2	(2)	–	–
Curtobacterium sp. SBUG 2094	0.0 ± 0.01	3	–	–
Curtobacterium sp. SBUG 2097	30.0 ± 9.8	4	–	–
Rhodococcus sp. SBUG 2075	0.0 ± 0.01	3	–	–
Rhodococcus sp. SBUG 2093	0.2 ± 0.04	3	–	–
Fusarium oxysporum SBUG-M 1727	45.6 ± 1.2	2	–	3
Fusarium sp. SBUG-M 1731	10.7 ± 8.0	2	–	1
Fusarium sp. SBUG-M 1732	0.2 ± 0.16	2	–	–
Lodderomyces elongisporus SBUG-Y 2196	50.6 ± 5.3	2	–	–
Pseudozyma aphidis SBUG-Y 2194	1.6 ± 0.75	4	–	–
Pseudozyma aphidis SBUG-Y 2195	5.2 ± 0.72	4	–	–
Pseudozyma sp. or *Sporisorium* sp. SBUG-Y 2193	1.0 ± 0.9	3	–	–
Purpureocillium lilacinus SBUG-M 1729	0.0 ± 0.02	3	–	–
Purpureocillium lilacinus SBUG-M 1730	0.0 ± 0.01	4	1	–
Rhodotorula mucilaginosa SBUG-Y 2201	0.2 ± 0.02	4	–	–
Sarocladium sp. SBUG-M 1728	3.3 ± 0.73	2	–	1
Yarrowia lipolytica SBUG-Y 2192	0.0 ± 0.00	5	–	–
Yarrowia lipolytica SBUG-Y 2204	0.0 ± 0.01	3	1	–
	Cyclohexanone	Cyclohexanols	ε-Caprolactone	Hexanedioic acid
Acinetobacter sp. SBUG 2088	57.0 ± 6.9	–	–	–
Candida parapsilosis SBUG-Y 2203	58.3 ± 7.9	2	–	1
Fusarium oxysporum SBUG-M 1733	88.4 ± 8.9	1	1	1
Fusarium solani SBUG-M 1735	30.6 ± 9.5	1	1	1
Fusarium sp. SBUG-M 1734	82.6 ± 1.9	–	–	–
Rhodotorula mucilaginosa SBUG-Y 2202	41.2 ± 0.4	3	1	1
	Pristane	2-Methyl-pentanedioic acid	4-Methyl-heptanedioic acid	4-Methyl-2-heptenedioic acid
Pseudomonas sp. or *Stenotrophomonas* sp. SBUG 2096	73.4 ± 5.9	–	–	–
Rhodococcus sp. SBUG 2031	4.3 ± 1.8	1	1	1
Rhodococcus sp. SBUG 2033	17.0 ± 1.0	1	–	1
	Biphenyl	Monohydroxybiphenyls[f)]	Phenyl-succinic acid	Benzoic acids
Pseudomonas sp. or *Stenotrophomonas* sp. SBUG 2091	60.0 ± 6.3	–	1	2[g)]
Pseudomonas sp. SBUG 2067	52.0 ± 4.2	1	1	1

[a)] All products were identified by HPLC-UV/Vis and/or GC-MS analyses as described in "Materials and methods"

[b)] Monocarboxylic acids: tetradecanoic, dodecanoic, decanoic, octanoic, and/or hexanoic acid

[c)] Dicarboxylic acids: hexanedioic acid

[d)] Ketones: 2-, 3-, and/or 6-tetradecanone

[e)] Data in parentheses are products formed only in traces

[f)] Monohydroxybiphenyls: 2-, 3-, or 4-hydroxybiphenyl

[g)] In addition to benzoic acid also 2,5-dihydroxybenzoic acid

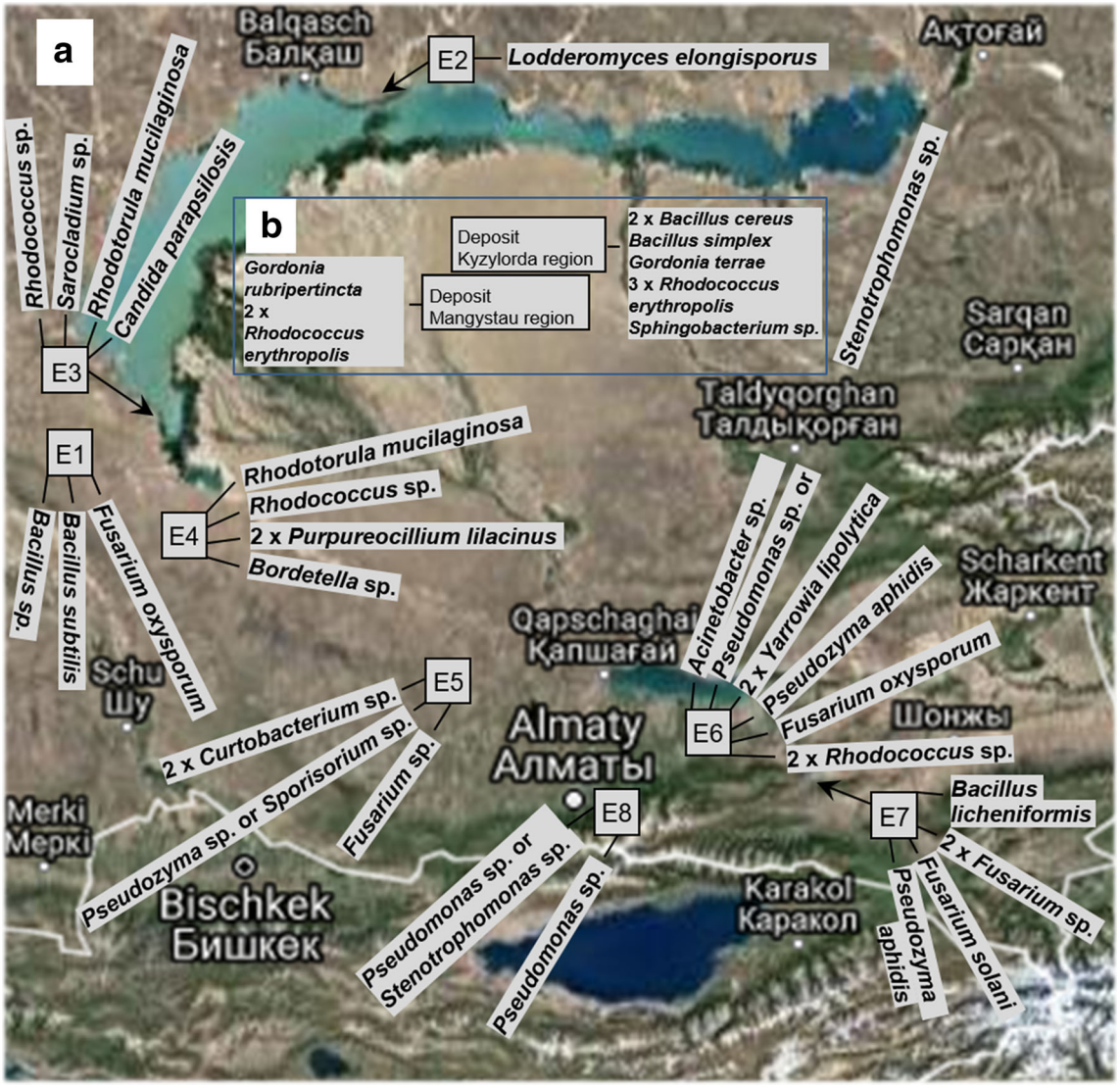

Fig. 2 Comparative presentation of the "culturome" biodiversity of the hydrocarbon degraders of the polluted soils (E1–E8) from the north bank of the Lake Balkhash to the Talgar Pass and of the oil-polluted deposits of the Mangystau and the Kyzylorda region (around 994 miles/1600 km and 466 miles/750 km further to the west)

attracted less attention in the field of aliphatic and alicyclic hydrocarbon degradation, were found in two different soil samples (E3 and E4) separated by around 56 miles/90 km in our studies in Kazakhstan.

Sixty-two percent of the 32 isolated species of 14 different genera belong to well-described hydrocarbon degraders like Bacilli and Rhodococci as well as to *Acinetobacter*, *Pseudomonas*, *Fusarium*, and *Candida* species. Strains from these genera have been isolated from polluted areas and investigated for decades (Annweiler et al. 2000; Colombo et al. 1996; Hassanshahian et al. 2012; Kachholz and Rehm 1978; Kästner et al. 1994; Long et al. 2017; Mohammadian et al. 2017; Raju et al. 2017). We extend the identified sources to include contaminated soils in Kazakhstan. Furthermore, we confirm their importance for the degradative power of the microbiome in polluted soils by showing that they are able to grow on the isolation substrates used (Fig. 1a, b), and to degrade *n*-alkanes, cycloalkanes, branched chain alkanes, and aromatics (Table 4).

Recently, we isolated and examined a variety of bacterial strains from two oil-polluted deposits in Kazakhstan. These isolates belong mostly to the genera *Gordonia* and *Rhodococcus* (using a wide range of hydrocarbon substrates), and to *Bacillus* (using aromatic hydrocarbons, especially PAH) (Mikolasch et al. 2015, 2016). In the current study, 56% of the powerful isolates are fungi and only 44% of the species are bacteria. Furthermore, we found several bacteria and fungi species that are able to transform multiple

hydrocarbons. The biodiversity of hydrocarbon degraders of the polluted soils from the north bank of the Lake Balkhash to the Talgar Pass as a whole (14 different genera) would seem to be higher than the diversity recovered from oil-polluted deposits (4 different genera) of the Mangystau and the Kyzylorda region (Fig. 2).

There are several recently published investigations characterizing the species diversity of microorganisms in petroleum-contaminated soils of oil fields (Mohammadian et al. 2017), crude oil sludge (Obi et al. 2016), or polluted soils (Long et al. 2017; Rajaei et al. 2013). In general, they focused either on bacteria or on fungi, while our studies include both bacteria and fungi and used multi-method analysis consisting of dry weight, HPLC-UV/Vis, and GC-MS measurements analyzing substrate consumption, biomass production, and metabolite formation.

Many authors, including ourselves, were able to isolate various promising bacteria and/or fungi for bioremediation purposes from contaminated samples. In contrast, the fungi from pristine and natural areas are less powerful in degradation of hydrocarbons (Martorell et al. 2017). For example, Martorell et al. (2017) reported that only one yeast strain from 105 was able to use *n*-alkanes as growth substrates. We, in contrast, isolated and characterized only 18 fungal strains from contaminated soil samples, but they are all potent hydrocarbon degraders, able to grow, and form detectable metabolites from hydrocarbon substrates. This indicates an adaptation of pristine areas to pollution. The samples collected on areas of petrol stations along the route from Lake Balkhash to the Talgar Pass in Kazakhstan suggest that these once-pristine areas adapted to oil pollution and hence permitted positive selection of species with the ability to degrade oil components. Such selection enables the removal of the pollutants by the local microorganisms and underlines the potential for self-regeneration of polluted areas.

Nevertheless, for acceleration of soil cleaning, heavily contaminated surfaces need to be restored either by feeding the native microbes with nutrients and/or other supplementary components (oxygen, biosurfactants, etc.) or—especially in case of scarcely degradable pollutants—by adding microorganisms with special degrading capabilities from strain collections or other habitats (Bento et al. 2005; Dua et al. 2002; Heipieper 2007; Juwarkar et al. 2010; Tyagi et al. 2011). This study illustrates the value of focused isolation efforts in times of increasing usage of cultivation-independent methods in applied and environmental microbiology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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