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EXPERIMENTAL ARTICLES

Identification of Hydrocarbon-Oxidizing *Dietzia* Bacteria from Petroleum Reservoirs Based on Phenotypic Properties and Analysis of the 16S rRNA and *gyrB* Genes

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Abstract—The taxonomic position of hydrocarbon-oxidizing bacterial strains 263 and 32d isolated from formation water of the Daqing petroleum reservoir (PRC) was determined by polyphasic taxonomy techniques, including analysis of the 16S rRNA and the *gyrB* genes. The major chemotaxonomic characteristics of both strains, including the IV type cell wall, composition of cell wall fatty acids, mycolic acids, and menaquinones, agreed with those typical of *Dietzia* strains. The DNA G+C content of strains 263 and 32d were 67.8 and 67.6 mol %, respectively. Phylogenetic analysis of the 16S rRNA gene of strain 32d revealed 99.7% similarity to the gene of *D. maris*, making it possible to identify strain 32d as belonging to this species. The 16S rRNA gene sequence of strain 263 exhibited 99.7 and 99.9% similarity to those of *D. natronolimnaea* and *D. cercidiphylli* YIM65002^T, respectively. Analysis of the *gyrB* genes of the subterranean isolates and of a number of *Dietzia* type strains confirmed classification of strain 32d as a *D. maris* strain and strain 263 as a *D. natronolimnaea* strain. A conclusion was made concerning higher resolving power of phylogenetic analysis of the *gyrB* gene compared to the 16S rRNA gene analysis in the case of determination of the species position of *Dietzia* isolates.

Keywords: actinobacteria, *Dietzia natronolimnaea, Dietzia maris*, taxonomy, petroleum reservoirs, 16S rRNA and *gyrB* genes, primers for amplification and sequencing of the *gyrB* genes in bacteria of the genus *Dietzia*

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The genes of type II topoisomerases, encoding the enzymes responsible for DNA topology, are among the promising taxonomic (phylogenetic) markers alternative to the 16S rRNA gene. Each of two homologous enzymes of this type (gyrase and topoisomerase IV) present in the bacterial genome consists of two subunits, and their overall size of 1200–1500 amino acid residues is statistically sufficient for phylogenetic analysis. The gene of gyrase beta subunit (*gyrB*) is most often used as a phylogenetic marker. For some groups of bacteria, the rate of *gyrB* horizontal transmission was shown to be the same as for the ribosome genes [1], and the rate of base pair replacement in the *gyrB* was found to correlate with the DNA–DNA hybridization data [2, 3]. Analysis of the *gyrB* sequence homology was successfully used to resolve the strains of such problematic genera as *Pseudomonas* [4, 5], *Bacillus* [6], *Acinetobacter* [7], *Gordonia* [8], and *Geobacillus* [9].

Dietzia is another of such problematic genera, with relatively low divergence of the 16S rRNA sequences between its species, which makes their phylogenetic identification difficult. The genus *Dietzia* presently consists of twelve species: *D. maris* [10, 11], *D. natronolimnaea* [12], *D. psychralcaliphila* [13], *D. kunjamensis* [14], *D. cinnamea* [15], *D. papillomatosis* [16], *D. schimae* and *D. cercidiphylli* [17], *D. lutea* [18], *D. aerolata* [19], *D. timorensis* [20], and *D. alimentaria* [21]. These bacteria have been isolated from soil, freshwater and saline environments, from the surface of fish and plants, and from clinical specimens. Some members of this species were reported to degrade aliphatic hydrocarbons and aromatic compounds and therefore may be recommended for bioremediation of petroleum-contaminated sites at low temperature and within a broad pH range [13, 22, 23]. The publications on corynebacteria from oilfields are scarce [24, 25], stressing the urgency of the present work.

The strains 32d and 263 were isolated from formation water of the Daqing oilfield on the medium with a

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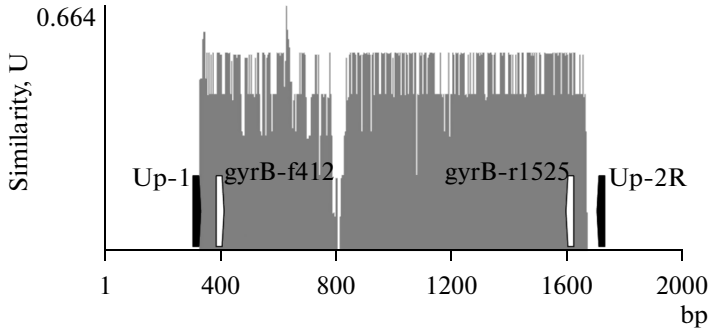

Fig. 1. Conservative nucleotide sequences of the *gyrB* gene of *Dietzia* strains and primer positions. Previously proposed primers [4] and the ones designed in the present work are highlighted black and white, respectively.

mixture of liquid C_{12}–C_{22} paraffins [26]. When grown on hydrocarbons, both strains produced surfactants and decreased the surface and interfacial tension of the culture medium [27]. Microbiological and physicochemical characterization of the Daqing oilfield and the data on the taxonomic position of the bacteria has been reported previously [26, 28]. Preliminary analysis of the 16S rRNA gene revealed phylogenetic relation of strains 32d and 263 and bacterial species *D. maris* and *D. natronolimnaea*, respectively. Based on its phenotypic properties and analysis of the complete 16S rRNA gene sequence, strain 32d was assigned to the species *D. maris*, while strain 263, which formed rough, mucous colonies, was tentatively assigned to the new species *'Dietzia daqingensis'* [Acc. nos. AY603001, AY603002].

The goal of the present work was to describe the physiological and biochemical properties and the taxonomic position of hydrocarbon-oxidizing *Dietzia* strains 32d and 263 using their phenotypic and chemotaxonomic characteristics and analysis of the 16S rRNA and *gyrB* genes.

MATERIALS AND METHODS

Bacterial strains. The strains 32d and 263 used in the work were isolated from the Daqing oilfield [26]. In 2003, the strains were deposited to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) under accession nos. DSM 44747 and DSM 44748, respectively. For comparative analysis, the following type strains were used: *D. psychralcaliphila* JCM10987^T and *D. natronolimnaea* JCM 11417^T from the Institute of Physical and Chemical Research (RIKEN), Wako, Japan, as well as *D. maris* DSM 43672^T and *Kocuria rosea* DSM 20477^T from DSMZ, Germany.

Cell morphology, Gram reaction, the spectrum of utilized substrates, gelatin, starch, and casein hydrolysis, formation of indole and sulfide, the methyl red test, DNA G+C content, DNA–DNA hybridization, and the composition of fatty acids were determined as described previously [29]. The cell wall chemotype was determined by a known method [30]. The composition of mycolic acids and isoprenoid quinones was determined as described previously [31, 32].

DNA isolation and gene amplification. DNA was extracted from single colonies grown on Plate Count Agar (PCA, Sigma, United States) using the Diatom^{tm}DNAprep reagent kit (Biokom, Russia) according to the manufacturer's recommendations with minor modifications. The 16S rRNA genes were amplified according to the standard procedure with the universal primers [33].

Amplification of the *gyrB* gene included two stages: (1) amplification with degenerate primers Up-1D and Up-2rD [4] and (2) reamplification of the PCR products with the primers Up-1sD_3 and Up-2srD, which are complementary to the 5'-region of degenerate primers Up-1D and Up-2rD, or with the specially constructed for *Dietzia* forward gyrB-f583_4 (5'-gatc CAC CMS ACS ATC CTS TAC TT-3') and reverse primers gyrB-r772 (5'-ctt Stc Scg Sgc gta Ytt gtt-3') in combination with the primers Up-1sD_3 or Up-2srD.

The primers Up-1sD_3, Up-2srD, gyrB-r772, gyrB-f583_4, as well as specially constructed for *Dietzia* the forward gyrB-f412 (5'-cgñgtcgaggtBSagatcaa-3') and reverse gyrB-r1525 (5'-gcaggatcgcctggaacat-3') primers were used for sequencing.

Primer selection and sequencing of the *gyrB* gene of *Dietzia* strains. The following primers were selected for identification of *Dietzia* using the nucleotide sequences of the *gyrB* gene encoding DNA gyrase: gyrB-f412 (5'-cgcgtcgaggtBSagatcaa-3') and gyrB-r1525 (5'-gcaggatcgcctggaacat-3'). Oligonucleotides complementary to the *gyrB* conservative sites were selected using aligned sequences of the 26 known *Dietzia gyrB* sequences (Fig. 1). The sequences were aligned using the CLUSTALW v. 1.75 software package, and the primers were selected manually. The thermodynamic parameters and the secondary structure of the primers were determined using Oligo Analyzer 3.1 (http://eu.idtdna.com/analyzer/applications/ oligoanalyzer/). The primer specificity was analyzed using the PrimerBLAST algorithm. The range of melting temperature (T_m) °C was 57.3–60.1°C (average T_m = 58.8°C), and for

Table 1. Primers and reaction protocols used for amplification and sequencing of the gyrase β-subunit gene (*gyrB*)

Forward primer	Reverse primer	Reaction conditions	Primer application
Up-1D	Up-2D	95°C—5 min; 2 cycles of: 95°C—30 s, 68–64°C—30 s, 72°C—1 min; 27 cycles: 95°C—30 s, 62°C—0 s, 72°C—1 min; 72°C—10 min	Amplification
Up-1SD_3	Up-2SrD	95°C—5 min; 30 cycles: 95°C—30 s, 55°C—30 s, 72°C—1 min; 72°C—10 min	Reamplification and sequencing
gyrB-f412	gyrB-r1525	95°C—3 min; 30 cycles: 95°C—30 s, 55°C—30 s, 72°C—40 s; 72°C —7 min	Amplification (for some strains) and sequencing
Up-1SD_3	gyrB-r772	95°C—5 min; 30 cycles: 95°C—30 s, 55°C—30 s, 72°C—40 s; 72°C—10 min	Reamplification and sequencing
gyrB-f583	Up-2SrD		
gyrB-f412	Up-2SrD		
Up-1SD_3	gyrB-r1525	95°C—5 min; 30 cycles: 95°C—30 s, 52°C—30 s, 72°C—0 s; 72°C —10 min	
Up-1SD_3	Up-2SrD	95°C—5 min; 30 cycles: 95°C—30 s, 55°C—30 s, 72°C—1 min; 72°C—10 min	

gyrB-r1525 T_m it was 57.9°C. The recommended annealing temperature was therefore $T_a = 55$°C. PCR was carried out under the following conditions: 95°C, 3 min; 30 cycles of: 94°C, 30 s, 55°C, 30 s, 72°C, 40 s; and 72°C, 7 min (Table 1). The primer position is shown on Fig. 1. These primers make it possible to amplify an extensive fragment (1113 bp) of the *gyrB* gene, including its most variable regions, which is required for bacterial identification.

The PCR products were sequenced on an ABI 3100 Avant Genetic Analyser (Applied Biosystems, United States) using the Dyenamic Terminator Cycle Sequencing Ready Reaction Kit (Amersham, United Kingdom).

Phylogenetic analysis. Primary analysis of the sequences was carried out using NCBI BLAST (www.ncbi.nlm.nih.gov/blast/). The sequences were aligned with those from the bank using CLUSTALX 2.0 [http://bips.u-strasbg.fr/fr/Documentation/ClustalX/]. Phylogenetic trees were constructed using the neighbor-joining algorithm implemented in the TREECONW software package [http://bioc-www.uia.ac.be/u/yvdp/treeconw.html]. Bootstrap analysis of 1000 alternative trees was used to determine the branching order (in %).

The sequences obtained in the work were deposited to GenBank under accession nos. AY603001, AY603002, AY360062, and KP192259 for the 16S rRNA genes, and under nos. KP192260–KP192263 for the *gyrB* genes.

RESULTS AND DISCUSSION

The Daqing oilfield is the largest oil deposit in China. It is located in the Heilongjiang province (PRC). The temperature of the studied oil-bearing horizons is 40–46°C. The oil contains hydrocarbons (87.4%), resins (11.5%), and bitumen (1%) and has specific gravity of 0.797 g/cm^3. The associated gas consists mainly of methane (87.5%) and its homologues (C_2–C_4) (8.3%), nitrogen (3.1%) and CO_2 (0.75%). The low-mineral (7.45 g/L) original formation water of the hydrocarbonate–sodium type usually contains no sulfate or sulfide and has pH 7.2–7.8 [28]. Sulfate concentrations in the water samples collected in the course of the present work were low (12–36 mg/L), while sulfide was detected in water from the near-bottom zone of injection well (8.5–10.0 mg/L). Prolonged exploitation with secondary water flooding was accompanied by inflow of dissolved oxygen and development of aerobic organotrophic and hydrocarbon-oxidizing bacteria in the oilfield. The numbers of aerobic bacteria in the near-bottom zone of injection wells and in the water from production wells were 10^4 and 10^2 cells/mL, respectively. Two strains designated

263 and 32d were isolated from formation water of production wells 11-5-263 and 2-32 [26].

Phenotypic characteristics of strains 32d and 263. Both strains formed red or coral-colored round colonies on solid PCA medium. Stain 263 formed both smooth and rough colonies, while all colonies of strain 32d were glossy. The bacteria were nonmotile cocci developing into short rods. No spores were formed. Gram reaction was positive.

Strains 263 and 32d, similar to the type strains of *D. natronolimnaea, D. psychralcaliphila*, and *D. maris*, were obligate aerobes. At neutral pH they grew on acetate, D-glucose, D-fructose, propionate, ethanol, butanol, peptone, yeast extract, and potato agar. D-galactose, lactose, L-arabinose, D-cellobiose, inositol, raffinose, rhamnose, D-ribose, salicin, D-sorbitol, xylose, D,L-lactate, methanol, isoleucine, methionine, threonine, tryptophan, and phenol were not used. Gelatin, casein, starch, and esculin were not hydrolyzed. Indole was not produced. The organisms were catalase-positive [10–13]. Strains 32d and 263 grew within a broad range of NaCl concentrations at the temperatures of 18–37 and 13–40°C, respectively, and were therefore well adapted to their habitat. It proved impossible to identify these *Dietzia* isolates based on their physiological characteristics alone. The differentiating features of strains 263 and 32d, as well as of the closely related *Dietzia* species, are listed in Table 2.

Chemotaxonomic characteristics of strains 32d and 263. The presence of $C_{16:0}$, $C_{17:0}$, $C_{18:1}\omega9c$, and 10-methyl-$C_{18:0}$ fatty acids is characteristic of the members of the genus *Dietzia* [19]. All five strains studied (263, 32d, *D. psychralcaliphila* JCM10987^T, *D. natronolimnaea* JCM 11417^T, and *D. maris* DSM 43672^T) had similar profiles of saturated fatty acids with predominance of $C_{16:0}$; strains 263, *D. maris* DSM 43672^T, and *D. natronolimnaea* JCM 11417^T also contained considerable amounts of the $C_{17:0}$ fatty acid (Table 3). Among unsaturated acids found in four strains were $C_{18:1}\omega9c$ and 10-methyl-$C_{18:0}$, typical of the *Dietzia* strains, while the type strain *D. maris* DSM 43672^T showed predominance of the $C_{18:1}\omega9c$ and $C_{17:1}\omega8c$ fatty acids.

Other chemotaxonomic characteristics of the isolates also indicated their affiliation with the genus *Dietzia*. The cell wall of strains 263 an 32d, as well as of the type strains, belonged to type IV and contained *meso*-diaminopimelic acid, arabinose, and galactose (Table 2) [30]. Mycolic acid chain in strain 263 contained 34–38 carbon atoms, as in *D. maris* and *D. natronolimnaea*. Isoprenoid quinones of *D. maris, D. natronolimnaea*, and *D. psychralcaliphila* type strains contain MK-8(H_2) menaquinone, while strains 32d and 263, apart from MK-8(H_2), contained MK-7. *Dietzia aerolata* is known to contain this menaquinone as a minor component [19].

Genomic characteristics of strains 32d and 263. The DNA G+C content of strains 263 and 32d was 67.8 and 67.6 mol %, respectively [26]. These values were close to those of other *Dietzia* strains (from 64.7 to 73.0 mol %) [17, 19]. DNA–DNA hybridization with the DNA of *Dietzia* type strains and one strain of the closely related genus *Kocuria* was carried out for identification of strains 32d and 263 (Table 4). For strain 32d, DNA–DNA hybridization levels with *D. maris* DSM 43672^T and *D. psychralcaliphila* JCM 10987^T were 77 and 30%, respectively, supporting its classification as a *D. maris* strain. DNA–DNA hybridization levels of strains 32d, DSM 43672^T, and JCM 10987^T with *K. rosea* DSM 20477^T were below 30%, differentiating clearly between these species and the genera *Deitzia* and *Kocuria*. The DNA–DNA hybridization level of strains 263 and *D. natronolimnaea* JCM 11417^T with other known *Dietzia* and *Kocuria* strains was too low for identification of these strains (Table 4). Importantly, the level of DNA–DNA hybridization between the type strain *D. cercidiphylli* YIM 65002^T and *D. natronolimnaea* CBS 107.95^T [17], as well as between *D. aerolata* Sj14a^T and the type strains *D. schimae* DSM 45139^T, *D. cercidiphylli* DSM 45140^T, and *D. maris* DSM 43672^T [19] was also low (27.8, 28, 19, and 26%, respectively). Other molecular genetic techniques were required for reliable identification of the isolates.

Comparative phylogenetic analysis of the 16S rRNA and *gyrB* for *Dietzia* strains. At the first stage of the study, preliminary assessment of resolving power of analysis of the *gyrB* sequences for phylogenetic identification of *Dietzia* species was carried out. For this purpose, the phylogenetic trees obtained by sequencing of the 16S rRNA and *gyrB* genes of 25 *Dietzia* strains, including the type strains of nine described species and two unidentified strains with both sequences stored in GenBank, were compared.

In general, topology of the trees was similar for both phylogenetic markers (Figs. 2 and 3). However, divergence between the 16S rRNA and *gyrB* gene sequences exhibited good correlation at the interspecies level and considerable differences at the interspecies one. Thus, the similarity between the 16S rRNA gene sequences was 99.9–100% for seven *D. maris* strains, 100% for ten *D. natronolimnaea* strains, and 99.8% for two *D. cinnamea* strains. For the *gyrB* genes, the similarity values were 95.6–99.0, 99.2–99.8, and 99.5%, respectively. At the same time, the similarity between the 16S rRNA gene sequences of these species was 97.6–99.6% and considerably exceeded the level for the *gyrB* genes (88.0–90.3%). This high divergence level of the *gyrB* sequences at the interspecies level provides for more accurate species identification of new *Dietzia* strains.

This may be demonstrated for members of other, still monotypic *Dietzia* species. The taxonomic status of *D. alimentaria* as an independent species was

Table 2. Differentiating characteristics of strains 263 and 32d isolated from oilfields and of the phylogenetically related *Dietzia* species

Feature	Strain 32d, DSM 44747	Strain 263, DSM 44748	*D. maris* DSM 43672^T	*D. natronolimnaea* JCM 11417^T	*D. psychralcaliphila* JCM10987^T
1	2	3	4	5	6
Cell morphology	Cocci to rods	Cocci to rods	Cocci to rods	Rods	Rods
Motility	–	–	–	–	–
Acid production from:					
Glycerol	–	–	+		–
Maltose		+	–	–	–
Mannose		+	–		–
Mannitol	+	–	–	+	–
Sucrose	+	+	–	–	–
Utilization of:					
n-Alkanes	C_{12}, C_{16}, C_{18}, C_{24}	C_{12}, C_{14}, C_{16}, C_{18}, C_{24}	C_6–C_{17}, C_{19}, C_{23}	C_6–C_{18}	C_{13}, C_{14}, C_{15}, C_{16}
Formate	+/–	–		–	
Butyrate	+	+	+		+
Valerate			+	+	+
Citrate	–	–	–	+	–
Benzoate	–	+	–		
Fumarate	+	+	+	+	–
Malate	+	+	+		–
Pyruvate	+	+	+	–	+
Succinate	+		+	+	–
L-Alanine	–	–	–	V	–
L-Arginine	+	+		+	–
L-Asparagine	+	+		+	–
L-Glycine	–	–		+	
Glutamate		–	+	+	–
Histidine		–	–	–	–
DL-Phenylalanine	–	+		+	–
L-Proline	+	–	–	+	–
L-Serine	+/–	–	+	+	
L-Valine	–	–		+	
$NO_3^- \rightarrow NO_2^-$			+	NA	–
Methyl red test	–	–	–	NA	–
H_2S formation	–	–	+*	NA	–
Urease	–	–	–	NA	–

Table 2. (Contd.)

Feature	Strain 32d, DSM 44747	Strain 263, DSM 44748	*D. maris* DSM 43672^T	*D. natronolimnaea* JCM 11417^T	*D. psychralcaliphila* JCM10987^T
1	2	3	4	5	6
Isoprenoid quinones	MK-8(H_2), MK-7	MK-8(H_2), MK-7	MK-8(H_2)	MK-8(H_2)	MK-8(H_2)
Mycolic acid chain length	ND	34–38	34–38	34–38	34–39
Cell wall type	IV	IV	IV	IV	IV
Cell wall contains:					
meso-Diaminopimelic acid	+	+	+	+	+
Arabinose	+	+	+	+	+
Galactose	+	+	+	+	+
NaCl range for growth, %	0–15	0–10	0–8	0–8	0–10
Growth in pH range		6–10.8		6–10	7–10
Optimal pH		9.3		9.0	9–10
Temperature range for growth, °C	18–37	13–40	20–42	10–45	5–30
DNA G+C content, mol %	67.6	67.8	73.2	66.1	69.6
References	Present work	Present work	10, 11	12	13

Designations: "+" and "–" stand for growth and its absence, respectively; V indicates variation among different strains; NA stands for not analyzed.

beyond doubt, since it formed a separate branch on both types of trees and the divergence of its 16S rRNA gene (97.4–99.2%) and *gyrB* (85.3–86.5%) sequences from the relevant genes of other *Dietzia* species was relatively high. Phylogenetic position of the type strains of *D. psychralcaliphila* and *D. kunjamensis* was less definite, since they formed a cluster together with *D. natronolimnaea* and *D. maris* strains with 99.7 and 99.9% similarity, respectively. On the gyrB tree, however, they formed separate branches with 92.2–92.7 and 95.1–95.7% similarity with the latter species, which confirmed their status as independent species. Unidentified strains *Dietzia* sp. W5004 and *Dietzia* sp. W5026, which had an indeterminate position on the 16S rRNA gene tree, formed a separate cluster on the gyrB tree with 99.6% similarity between the strains and 87.4–91.8% similarity to other species, which may suggest their status as a new *Dietzia* species.

The situation with *D. cercidiphylli* and *D. schimae* was opposite. On the 16S rRNA gene-based tree, *D. cercidiphylli* and *D. schimae* fell into the clusters of the species *D. natronolimnaea* and *D. maris* with the similarity of 99.9 and 99.9%, respectively; their position of the gyrB tree remained the same with high similarity levels (99.4–100 and 98.0–98.7%, respectively). Thus, according to these results, the status of *D. cercidiphylli* and *D. schimae* as independent species causes certain doubt. It should be noted that the results of DNA–DNA hybridization played an important part in description of new species in these pairs [17]. However, as was suggested in our work on the genus *Geobacillus* [9], these data may be prone to experimental errors due to the differences in reaction conditions and to the absence of adequate control values at the inter- and interspecies levels.

Thus, comparative analysis of the phylogenetic relations within the genus *Dietzia* makes it possible to recommend *gyrB* gene as phylogenetic markers with the highest resolving power for determination of the species structure of this genus.

Identification of new *Dietzia* isolates based on analysis of the 16S rRNA and *gyrB* gene sequences. To specify the phylogenetic positions of strains 263 and 32d, as well as of the third strain, 26/10-1, also originating from an oilfield, recently isolated in pure culture, and tentatively identified as a *Dietzia* strain, their *gyrB* sequences were determined. For this purpose, the newly designed primers specific for *gyrB* amplification in *Dietzia* strains were applied.

Experimental testing of the primers was carried out using genomic DNA of five *Dietzia* strains—type strains *D. psychralcaliphila* DSM 44820^T and

Table 3. Composition of cell wall fatty acids for the oilfield strains 263 and 32d and for the phylogenetically related *Dietzia* type strains

Fatty acids	Strain 32d, DSM 44747	Strain 263, DSM 44748	*D. maris* DSM 43672^T	*D. natronolimnaea* JCM 11417^T	*D. psychralcaliphila* JCM 10987^T
Saturated fatty acids					
$C_{14:0}$	1.31	1.08	0.89	1.07	0.72
$C_{15:0}$	2.20	1.35	9.89	1.43	1.57
$C_{16:0}$	**23.63**	**25.77**	**19.26**	**28.09**	**29.15**
$C_{17:0}$	2.70	7.56	**12.34**	8.88	4.08
$C_{18:0}$	2.28	3.93	6.56	2.96	2.12
$C_{19:0}$		5.12	0.76	0.78	2.36
Unsaturated fatty acids					
$C_{16:1}\omega7c$	5.33	5.38	6.39	8.63	3.01
$C_{16:1}\omega9c$	10.95	2.68	1.42	2.29	6.48
$C_{17:1}\omega8c$	7.65	1.35	**13.89**	2.73	4.90
$C_{18:1}\omega7c$		1.08			1.49
$C_{18:1}\omega9c$	**25.82**	**12.12**	**14.05**	**17.46**	**14.60**
$C_{19:1}$		3.77	1.32	1.18	1.21
10-Methyl fatty acids					
10Me-$C_{16:0}$	0.60	0.54	0.45	0.60	1.34
10Me-$C_{17:0}$	1.23	0.98	4.87	0.70	1.62
10Me-$C_{18:0}$	**15.47**	**27.29**	**7.91**	**23.20**	**25.35**
Others	0.83				
Total, %	100	100	100	100	100

Table 4. DNA G+C content and DNA–DNA hybridization levels of the subterranean isolates with related *Dietzia* species and other corynebacteria

Genus, species, strain	DNA G+C content, mol %	Hybridization with the DNA of strains:					
		1	2	3	4	5	6
1. Strain 263, DSM 44748	67.8	100					
2. Strain 32d, DSM 44747	67.6	28	100				
3. *D. maris* DSM 43672^T	73.2[1]	31	77	100			
4. *D. psychralcaliphila* JCM 10987^T	69.6[2]	33	30	30	100		
5. *D. natronolimnaea* JCM 11417^T	66.1[3]	15	21	24	26	100	
6. *K. rosea* DSM 20477^T	70.2[4]	19	27	29	25	15	100
7. *E. coli* K 12	51.7						

[1]—Data from [11]; [2]—data from [13]; [3]—data from [12]; [4]—data from [34].

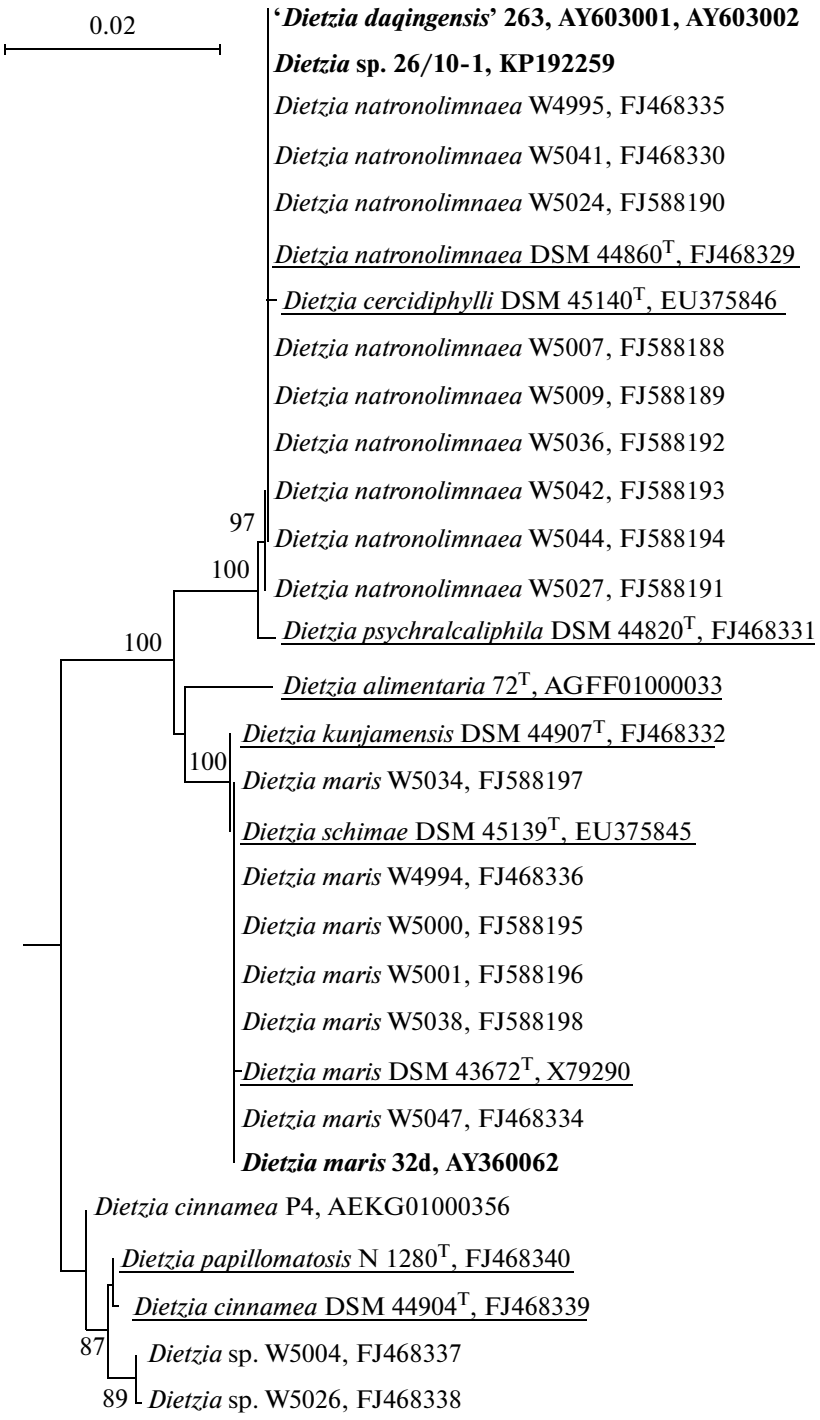

Fig. 2. Phylogenetic tree of the genus *Dietzia* constructed based on the 16S rRNA gene sequencing. The tree was constructed using the neighbor-joining algorithm with the sequence of *Nocardia acidivorans* as an outgroup. Type strains of the species are marked by underlining. The new oilfield strains are marked by boldface. The numerals indicate the reliability of the branching order determined by bootstrap analysis, with the values exceeding 85% accepted as significant. The scale bar shows the evolutionary distance corresponding to 2 replacements per 100 nucleotides.

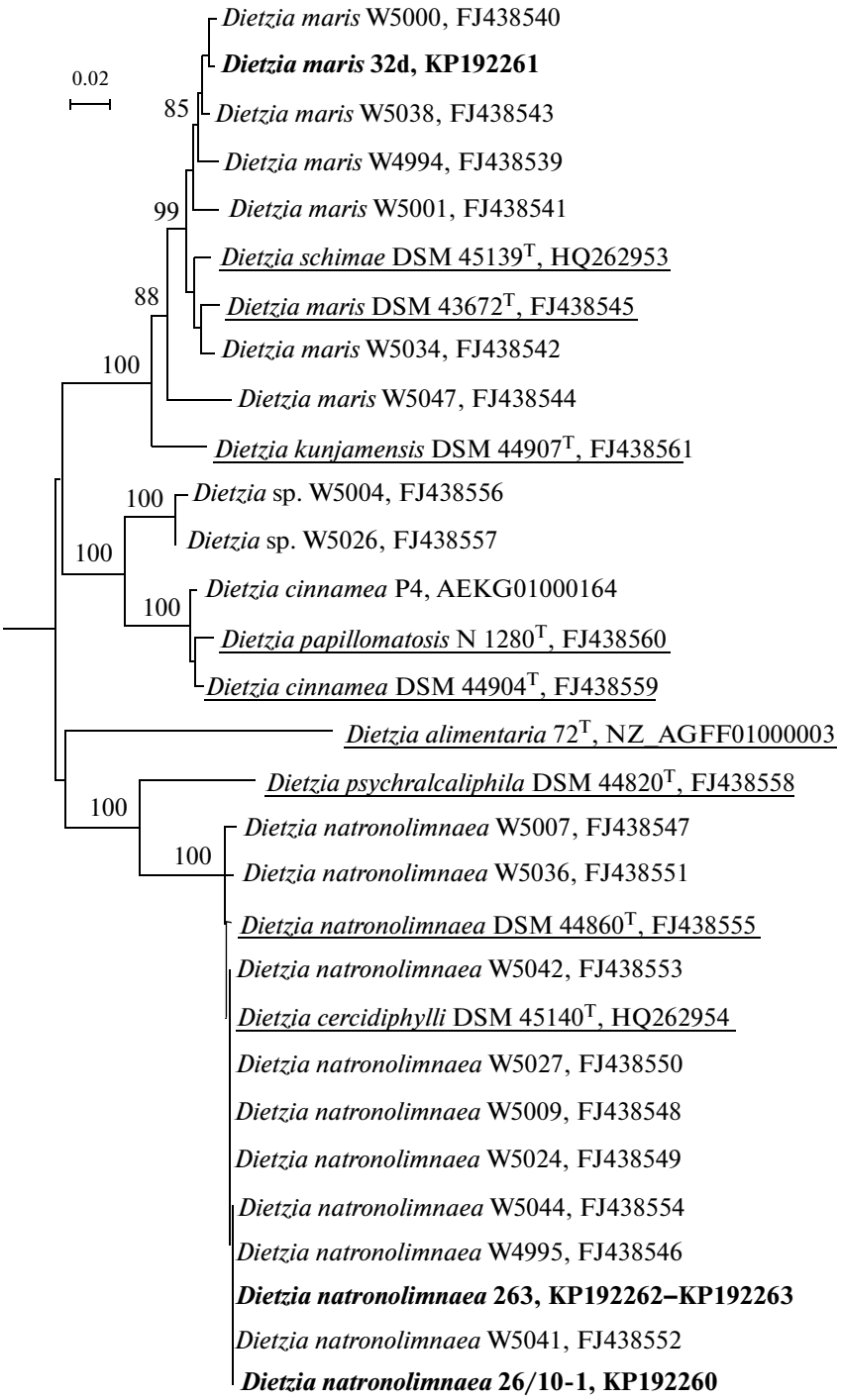

Fig. 3. Phylogenetic tree of the genus *Dietzia* constructed based on *gyrB* gene sequencing. The tree was constructed using the neighbor-joining algorithm with the sequence of *Nocardia acidivorans* as an outgroup. Type strains of the species are marked by underlining. The new oilfield strains are marked by boldface. The numerals indicate the reliability of the branching order determined by bootstrap analysis, with the values exceeding 85% accepted as significant. The scale bar shows the evolutionary distance corresponding to 2 replacements per 100 nucleotides.

D. natronolimnaea DSM 44860^T, which acted as the positive controls, as well as DNA of the new *Dietzia* strains, 32d, 26/10-1, and 263 (with two colony variants, smooth and rough). In all cases, the PCR product of expected length was obtained (Table 5).

Sequencing of the amplified fragments (~1000 bp), which corresponded to the *Dietzia gyrB* genes according to BLAST analysis. The *gyrB* fragments of the type strains of *D. psychralcaliphila* and *D. natronolimnaea*, which were used as the positive controls, were identi-

Table 5. Phylogenetic relationships between the 16S rRNA genes and the *gyrB* genes of the oilfield strains and the relevant genes of related *Dietzia* type strains

No.	Species, strain	Phylogenetically related strain according to the 16S rRNA gene sequencing [accession no., length, bp]	Identity of the 16S rRNA genes, %	Phylogenetically related strain according to the *gyrB* gene sequencing [accession no., length, bp]	Identity of the *gyrB* genes, %
1	*D. psychralcaliphila* JCM 10987^T	*D. psychralcaliphila* JCM 10987^T [AB159036, 1439 bp]	100	*D. psychralcaliphila* DSM 44820^T [FJ438558.1, 1307 bp]	100
2	*D. natronolimnaea* JCM 11417^T	*D. natronolimnaea* DSM 44860^T [NR_116683, 1439 bp]	100	*D. natronolimnaea* DSM 44860^T [FJ438555.1, 1286 bp]	100
3	263, DSM 44748, smooth colonies	*D. natronolimnaea* CA161 [GQ870426, 1433 bp]	100	*D. natronolimnaea* W5036 [FJ438551.1, 1230 bp]	99
4	263, DSM 44748, rough colonies	*D. natronolimnaea* CA161 [GQ870426, 1433 bp]	100	*D. natronolimnaea* W5036 [FJ438551.1, 1230 bp]	99
5	32d, DSM 44747	*D. maris* 41 [KF923451, 1483 bp]	100	*D. maris* W5038 [FJ438543.1, 1290 bp]	99
6	26/10-1	*D. natronolimnaea* NF047 [AB298545, 1511 bp]	100	*D. natronolimnaea* W5009 [FJ438548, 1324 bp]	100

cal (100% similarity) to the relevant fragments from the GenBank database, confirming the applicability of the newly designed primers for amplification and sequencing of the *gyrB* genes of *Dietzia* strains.

According to the results of the 16S rRNA gene sequencing, the strain *Dietzia* sp. 32d belonged to the cluster of *D. maris* strains with 99.9% similarity, which supported its tentative assignment to this species. On the gyrB tree, this strain occupied a similar position inside the cluster of *D. maris* strains with a high level of similarity (96.0–99.3%), corresponding to the intraspecies level for the strains of this species. These data therefore confirmed the taxonomic status of *Dietzia* sp. 32d as a *D. maris* strain.

The smooth and rough colonies of the strain *Dietzia* sp. 263 had identical sequences of both the 16S rRNA gene and *gyrB* (100% similarity). Both at the 16S rRNA gene and at the *gyrB* gene level, the strain *Dietzia* sp. 263 was located within the cluster of *Dietzia natronolimnaea* strains with a high level of sequence homology (100 and 99.4–99.7%, respectively). Its tentative identification as a member of a new species *"Dietzia daqingensis"* was therefore not confirmed. Strain 26/10-1 was also located within the cluster of *Dietzia natronolimnaea* strains with high similarity of both the 16S rRNA genes (100%) and *gyrB* (99.0–99.5%), which supports its classification as a strain of this species.

Our results confirm wide occurrence of coryneform hydrocarbon-oxidizing bacteria of the genus *Dietzia* in oilfields and their adaptation to this environment. Since good applicability of the *gyrB* analysis for identification of new *Dietzia* members was demonstrated, the *gyrB* gene may be recommended as a phylogenetic marker with the highest resolving power for determination of the species structure of the bacterial genus *Dietzia*.

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