Characterization of a Novel Long-Chain *n*-Alkane-Degrading Strain, *Dietzia* sp. E1

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Z. Naturforsch. **65c**, 693–700 (2010); received July 19/September 3, 2010

The newly isolated strain E1, identified as a *Dietzia* sp., proved to have an excellent ability to degrade *n*-C_{12} to *n*-C_{38} alkane components of crude oil. The preferred substrate was the very long-chain alkane *n*-eicosane at an optimal temperature of 37 °C and an optimal pH of 8 under aerobic conditions. The growth and substrate uptake kinetics were monitored during the *n*-alkane fermentation process, and *Dietzia* sp. E1 cells were found to possess three distinct levels of cell-surface hydrophobicity. Gas chromatographic/mass spectrometric analysis revealed that intracellular substrate mineralization occurred through the conversion of *n*-alkane to the corresponding *n*-alkanal. The monoterminal oxidation pathway was presumably initiated by AlkB and CYP153 terminal alkane hydroxylases, both of their partial coding sequences were successfully detected in the genome of strain E1, a novel member of the *Dietzia* genus.

Key words: n-Alkane, *Dietzia*, Cell-Surface Hydrophobicity

Introduction

Crude oil spills apparently cause enormous ecological problems in marine and land environments worldwide. Considering that long-chain *n*-alkanes represent the main components of the pollutions, the investigation of microbes having the ability to degrade these compounds is crucial. As surveyed by Wentzel *et al*. (2007), several isolates can degrade alkanes ranging from *n*-C_{12} to *n*-C_{36}, and approximately half of them belong to high-G+C Gram-positive mycolic acid-containing actinomycetes. Among them, mainly *Rhodococcus*, *Nocardia*, and *Gordonia* spp. (Hamamura *et al*., 2006; Baek *et al*., 2007; Quatrini *et al*., 2008) are suspected to play a major role in crude oil bioremediation, however, some *Dietzia* spp. have also been detected in different hydrocarbon-contaminated ecosystems recently (Chaillan *et al.*, 2004; Brito *et al.*, 2006; Radwan *et al.*, 2007; Sette *et al.*, 2007). Many of these polluted habitats are saline, which may promote the spreading of the halophilic, *n*-alkane-catabolizing *Dietzia* spp. (Riis *et al.*, 2003; Kleinsteuber *et al.*, 2006; Al-Awadhi *et al.*, 2007). Although the *Dietzia* genus was established only in 1995, eleven type strains have already been reported, six of them in the last three years. Some of the type strains were reported to mineralize *n*-alkanes: *D. maris* DSM 43672^T, *n*-C_6 to *n*-C_{23} alkanes (Rainey *et al.*, 1995); *D. psychralcaliphila* DSM 44820^T, *n*-C_{13} to *n*-C_{24} alkanes (Yumoto *et al.*, 2002); and *D. natronolimnaea* DSM 44860^T, paraffin (Yassin *et al.*, 2006). Crude oil degradation by two other individual pure cultures has also been described: *D. cinnamea* strain P4 degraded *n*-C_{11} to *n*-C_{36} alkanes (von der Weid *et al.*, 2007), whilst *Dietzia* sp. A14101 depleted *n*-C_6 to *n*-C_{26} alkanes (Bødtker *et al.*, 2009).

As illustrated in the examples above, some *Dietzia* spp. have the ability to deplete medium- or long-chain *n*-alkanes, however, no experimental evidence is found in the literature as concerns the effects of environmental parameters on hydrocarbon biodegradation rates, the alkane adhesion mechanisms, the catabolic pathways, and the class of genes responsible for *n*-alkane degradation in this genus. The present paper reports these comprehensive physiological properties for a novel *Dietzia* isolate having special *n*-alkane catabolic features.

0939–5075/2010/1100–0693 $ 06.00

Material and Methods

Isolation and identification of isolate E1

In order to isolate long-chain *n*-alkane-degrading bacteria, 1 g of different petroleum-contaminated Hungarian soil samples were rinsed in phosphate-buffered saline (PBS), pH 7.2. 0.5-ml aliquots of the liquid phases were injected into 50 ml of high nitrogen-phosphorus-sulfur (HNPS) minimal medium (Leahy *et al.*, 2003) supplemented with 1 g/l purified solid paraffins (C_{16}–C_{40} fraction), and the flasks were incubated aerobically at 30 or 37 °C. After 168 h, fractions of the cultures were serially diluted and spread on Luria-Bertani (LB) plates (Sambrook *et al.*, 1989). One isolate, designated E1, was identified and used for further studies. 16S rDNA analysis was performed by colony PCR using the EubB(27F)/EubA(1522R) primer pair and the PCR conditions described by Suzuki and Giovannoni (1996). The phenotypic characterization was carried out in accordance with Mayilraj *et al.* (2006).

Cultivation conditions

Dietzia sp. E1 cultures were maintained at 37 °C in GPY complete medium (10 g/l glucose, 10 g/l peptone, 6 g/l yeast extract). In *n*-alkane degradation experiments, E1 cells were routinely cultivated in 100-ml Erlenmeyer flasks containing 50 ml of modified HNPS (MNPS) minimal medium [0.1 M Na-phosphate buffer, 5 g/l $(NH_4)_2SO_4$, 5 g/l KCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $CaCl_2 \cdot 2H_2O$, 10 ml/l trace element solution SL-4 (http://www.dsmz.de/microorganisms/media_list.php, see Medium 14 and 27), pH 8.0] supplemented with 1 g/l *n*-hexadecane or *n*-eicosane. For inoculation, overnight GPY-grown E1 starter cultures were applied. The centrifuged (16,000 × *g*, 5 min) cells were resuspended in MNPS minimal broth and were diluted to a starting cell number of 10^6/ml. Flasks were incubated at 37 °C at 200 rpm for 16–20 h.

To evaluate the effect of the pH value on the E1 cell growth, 0.1 M Na-phosphate-buffered (pH 7–10) GPY or 1 g/l *n*-hexadecane-amended MNPS media were applied. The influence of temperature and salinity on *n*-hexadecane depletion was investigated within the ranges 22–44 °C and 0–20% NaCl. For determination of the utilizable hydrocarbon spectrum, 1 g/l heavy crude oil (Demjén-7 oil well, MOL Hungarian Oil and Gas Plc., Budapest, Hungary), the individual *n*-alkane (with a chain length from C_6 to C_{40}), pristane, different aromatic hydrocarbons or 2.9 g/l sodium acetate were added as carbon source. Microbial growth was monitored via the increases in optical density at 600 nm (OD_{600}) and microscopically counted total cell number. All measurements were performed in triplicate.

Analytical procedures

Alkane degradation by strain E1 was analysed by gas chromatography (GC). Cultures were extracted with chloroform/methanol (3:1) (Koma *et al.*, 2001). The organic phase was spiked with 1,4-dichlorobenzene as internal standard (40 mg/l), and 2 ml of the organic phase were injected into an HP-5890 Series II GC instrument fitted with an HT-5 capillary column (SGE 25 m x 0.32 mm x 0.1 mm) and an FID detector. The conditions of splitless analysis were as follows: injector and detector temperature, 300 °C; column temperature, 45 °C for 1 min, then increased to 280 °C at a rate of 15 °C/min and held at 280 °C for 10 min.

For identification of intermediates of the alkane biodegradation pathway, the strain was cultivated in 50 ml of MNPS minimal medium supplemented with 1 g/l of *n*-hexadecane. Cells were harvested in the early stationary phase (OD_{600} = 1.45) and washed twice with physiological saline solution. The resuspended cells were sonicated on ice for 1.5 min 3 times at 25 W (Vibracell, Sonics & Materials, Newtown, USA). The total crude extract was acidified to pH 2, and it was further extracted with an equal volume of diethyl ether. 2 μl of the organic layer were analysed directly (without methanolysis) on an Agilent 6890N GC/5975 MS instrument fitted with an HP-5MS capillary column (30 m x 0.25 mm x 0.25 mm) in the splitless mode. The conditions of analysis were as follows: inlet temperature, 300 °C; column temperature, 50 °C for 1 min, then increased to 260 °C at a rate of 20 °C/min, then to 300 °C at a rate of 30 °C/min and held for 30 min.

The microbial adhesion to hydrocarbon (MATH) test was made with a Unicam Helios α spectrophotometer according to Baldi *et al.* (1999). *Dietzia* sp. E1 was cultivated on *n*-hexadecane carbon source as described above. Cells were harvested at regular time intervals by centrifugation (16,000 × *g*, 10 min), washed twice with deionized water, and suspended in PBS to

obtain a final absorbance at 600 nm of 0.4 to 0.6 (A_0). Triplicate aliquots (1 ml) of the suspensions were supplemented with 50 µl *n*-hexadecane to extract the hydrophobic cells. After 1 min vortexing, the absorbance of the aqueous phase (A_t) was measured, and the relative hydrophilicity values of cells were expressed as $A_t/A_0 \cdot 100$.

DNA manipulations

In order to detect the presence of AlkB-type and CYP153-type alkane hydroxylase (monooxygenase) sequences in strain E1, their genes fragments were amplified in PCR reactions using the specific degenerate primer pairs TS2S/deg1RE and P450fw1/P450rv3 and PCR methods described by Smits *et al.* (1999) and van Beilen *et al*. (2006), respectively. Chromosomal DNA of strain E1 prepared as described elsewhere (Szvetnik *et al.*, 2010) served as template for the reactions. The PCR products with the expected size were isolated from 1% agarose gel and were purified with the EZ-10 Spin Column DNA Extraction Kit (Bio Basic Inc., Markham, Canada). Fragments were then ligated into the *Sma*I site of pBluescript II SK(+) (Stratagene, La Jolla, USA), and cloned into *E. coli* DH5α (Invitrogen, Carlsbad, USA); 10 ampicillin-resistant colonies were randomly chosen from each transformation, and both strands of the inserts were sequenced using the M13F(-41) and M13R(-48) primers.

Results

Isolation and identification of strain E1

Our overarching goal was to isolate effective long-chain *n*-alkane-degrading bacteria. For this purpose polluted habitats were screened: HNPS minimal medium supplemented with 1 g/l of purified paraffins as sole carbon source was inoculated with soil extracts and incubated at 30 or 37 °C under aerobic conditions. The paraffin-utilizing culture exhibiting the highest colony-forming unit on LB plates was chosen, and the dominant, reddish-orange pigment-producing isolate (E1) was used for further studies. Cell counting results suggested that this strain had a greater hydrocarbon-degrading ability at 37 °C than at 30 °C.

The hydrocarbon-degrading bacterium E1 was classified by 16S rDNA analysis and phenotypic characterization. The PCR-amplified and sequenced 1404-bp 16S rRNA gene was reported in the GeneBank database under accession number DQ011667. It displayed the highest similarity (99.86% and 99.64% identity) to that of *Dietzia* sp. 158Xa1 (EU090135) and the type strain *D. cinnamea* DSM 44904^T (AJ920289), respectively. E1 cells are aerobic, Gram-positive, non-motile, non-spore-forming rods. The colonies are circular, convex, and glistening. The catalase, oxidase, and nitrate reduction reactions are positive, whereas the tests for H_2S production, lipase, lecithinase, ornithine decarboxylase, starch hydrolysis, the Voges-Proskauer test, and the methyl red test are negative. This strain utilizes salicylate, citrate, acetate, propionate, and L-arginine, but not lactate, L-alanine, L-histidine or L-lysine. No acid production was observed from glucose, maltose, xylose, lactose, ribose, raffinose, arabinose, rhamnose, melibiose, fucose, sorbose, cellobiose, sucrose, myo-inositol or mannitol.

In contrast with the 16S rDNA analysis, the phenotypic characterization suggests the closer relatedness of strain E1 to *D. maris* DSM 43672^T and *D. natronolimnaea* DSM 44860^T (data not shown) rather than to *D. cinnamea* DSM 44904^T. On the basis of these findings, the isolate was finally assigned as *Dietzia* sp. E1, and it was deposited in the Hungarian National Collection of Agricultural and Industrial Microorganisms as NCAIM B02192.

Effects of cultivation conditions on microbial growth

The impact of the pH value on *Dietzia* sp. E1 cell growth during the utilization of *n*-hexadecane as model substrate or of GPY complete medium was investigated at 37 °C initially. After incubation for 16 h, the highest cell numbers were achieved at pH 9 on the complete medium, whilst the optimum pH for growth on *n*-hexadecane as carbon source was found to be around pH 8 (Fig. 1). Accordingly, the pH value of the MNPS minimal medium was set to pH 8, and this was applied throughout the study. The temperature optimum for growth on *n*-hexadecane as substrate proved to be within the range of 34–37 °C (data not shown). The subsequent investigations were therefore performed at 37 °C. Interestingly, the presence of 5, 10, 15 or 20% NaCl in the MNPS broth inhibited the *n*-alkane mineralization completely.

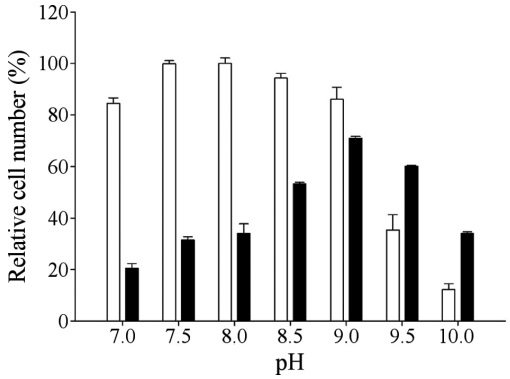

Fig. 1. Effect of pH value on E1 cell growth. Cells were grown aerobically for 16 h on either GPY complete medium (black columns) or on MNPS minimal broth supplemented with 1 g/l *n*-hexadecane (empty columns). The starting cell number was 10^6 cells/ml. The presented values are total cell number percentages relative to the maximum cell number (approximately $7.1 \cdot 10^8$ cells/ml) attained on *n*-C_{16} alkane as carbon source at 37 °C and pH 8.

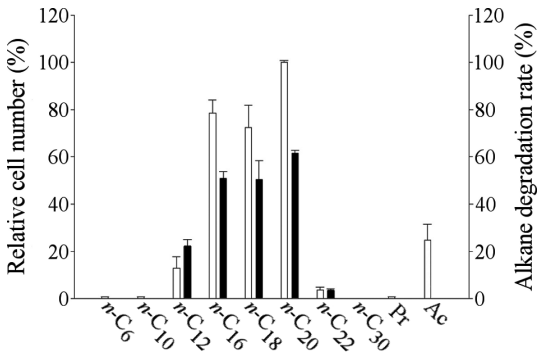

Fig. 2. Substrate spectrum of alkane degradation by *Dietzia* sp. E1. Cells were grown aerobically for 16 h on MNPS minimal broth supplemented with 1 g/l of different *n*-alkanes, pristane (Pr) or Na-acetate (Ac) at 37 °C and pH 8. The starting cell number was 10^6 cells/ml. The empty columns denote total cell number percentages relative to the maximum cell number (approximately $9.8 \cdot 10^8$ cells/ml) attained on *n*-C_{20} alkane as carbon source. The alkane degradation rate (black columns) denotes the amount of alkane consumed relative to the initial amount of added alkane substrate. Values were calculated from the results of triplicate GC measurements.

Determination of the hydrocarbon-degrading ability

The substrate specificity of *Dietzia* sp. E1 was studied using different hydrocarbon substrates. Neither monoaromatic and polyaromatic hydrocarbon compounds, nor the isoalkane pristane promoted the growth, but strain E1 catabolized individual *n*-C_{12}, *n*-C_{16}, *n*-C_{18}, *n*-C_{20}, *n*-C_{22} alkanes and their presumed late degradation intermediate, acetate, under the conditions tested. The *n*-C_{20} alkane was found to be the most appropriate of the examined substrates for growth and was depleted to the greatest extent (61.5%), as demonstrated by GC measurements (Fig. 2). During consumption of a complex carbon source, heavy crude oil, isolate E1 also used >*n*-C_{22} alkanes; the *n*-C_{38} alkane was the largest compound that underwent a significant decrease in concentration (Fig. 3).

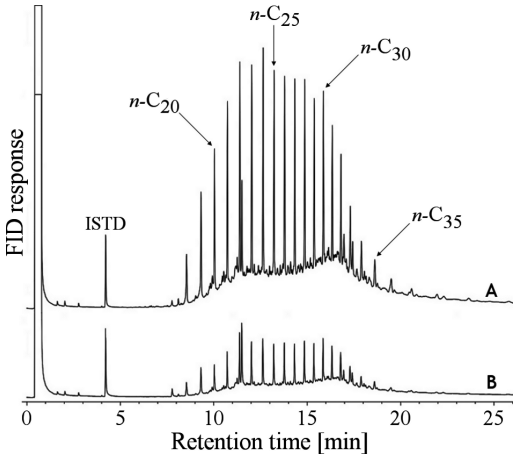

Fig. 3. Effect of complex substrate on utilization of *n*-alkanes. The gas chromatograms indicate the alkane substrates remaining in (A) 48-h abiotic control and (B) E1-inoculated cultures. The flasks contained MNPS minimal broth supplemented with 1 g/l heavy crude oil and were incubated aerobically at 37 °C and pH 8. The starting cell number of *Dietzia* sp. E1 was 10^6 cells/ml; ISTD, internal standard.

The n-alkane adhesion and degradation mechanism of Dietzia sp. E1

To become accessible, alkane substrates have to penetrate the cell membrane. This phenomenon frequently coincides with the change of cell-surface hydrophobicity, which can be monitored throughout the growth kinetics by the so-called MATH test. We previously found that E1 cells have a moderately hydrophobic surface when grown in GPY complete broth. The MATH test revealed that 14.3% of the cells can not be extracted by alkanes. After inoculation of this GPY-grown exponential-phase starter culture to *n*-C_{16} model substrate, the surface of E1 cells becomes

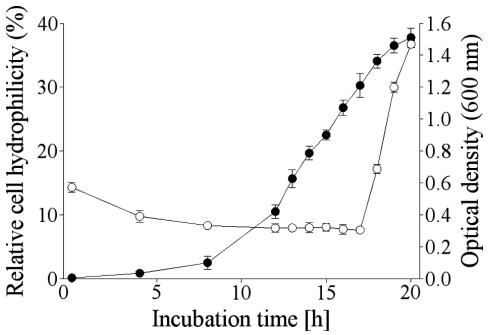

Fig. 4. Growth (filled symbols) and cell hydrophilicity (open symbols) kinetics of *Dietzia* sp. E1 cells grown in 50 ml of MNPS minimal broth supplemented with 1 g/l *n*-hexadecane at 37 °C and pH 8. The starting cell number was 10^6 cells/ml.

more and more hydrophobic during the early and middle exponential phase of the growth kinetics. To the end of the exponential phase, only 7.6% of the cells are unable to adhere to alkanes. The measured relative cell hydrophilicity as a function of the growth phase is displayed in Fig. 4. When cells entered the late exponential phase ($OD_{600} \approx$ 1.3), a dramatic increase in cell hydrophilicity was measured, reaching the relative hydrophilicity value of 36.7%. This physiological phenomenon correlated with the weakening of the interaction between the cell and *n*-hexadecane droplet surfaces. The substrate release was observed microscopically (figure not shown), and these hydrophilic cells were found to be easily separable from the surface of the remaining growth substrate by slight centrifugation (2,500 × *g*, 10 min).

In order to detect the bioavailable alkane substrate and to describe its degradation pathway, hydrophobic compounds from early stationary phase free E1 cells were extracted and analysed by GC-MS. As illustrated in Fig. 5, not only the intracellular growth substrate, *n*-hexadecane, but also its oxidation intermediate *n*-hexadecanal were successfully identified in the cell extract in commensurable amounts. The presented results suggest that *Dietzia* sp. E1 degrades *n*-alkanes via the monoterminal oxidation pathway.

Detection of alkane hydroxylase genes

Monoterminal alkane catabolism can be initiated by multiple enzymes. In case of another actinomycete strain, *Rhodococcus erythropolis* NRRL B-16531, the presence of four *alkB*-encoded integral membrane alkane hydroxylase (Whyte *et al.*, 2002) and two CYP153-type cytochrome P450 alkane hydroxylase paralogs (van Beilen *et al.*, 2006) were found. Unfortunately, no similar genetic analysis concerning *Dietzia* spp. has been published in the literature yet. Nevertheless, direct submissions relating to partial *alkB* and CYP153 genes of *Dietzia* sp. H0 (FJ435354) and *Dietzia* sp. H0B (FJ435355) have been recently reported in the GenBank database. Both strains appear to

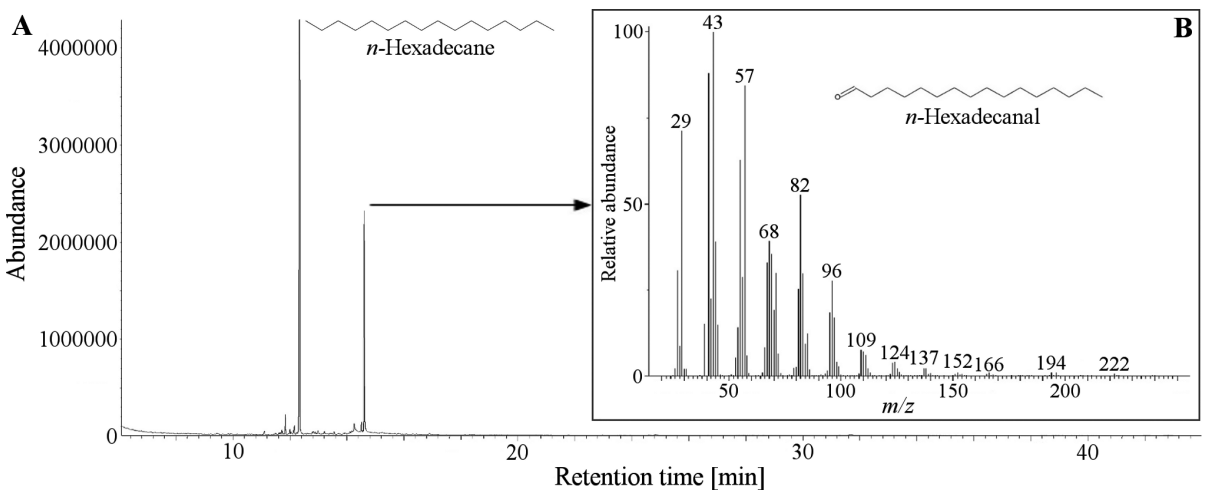

Fig. 5. GC-MS analysis of total crude extract of early stationary-phase *Dietzia* sp. E1 cells grown on *n*-hexadecane carbon source. (A) Total ion chromatogram; (B) mass spectrum of the 14.604-min retention time peak, identified as *n*-hexadecanal.

Table I. The effect of *n*-alkane exposure on cell-surface hydrophobicity in different bacteria.

Strain	Water-soluble substrate	After exposure to *n*-alkanes in	
		exponential phase	stationary phase
Acinetobacter venetianus VE-C3[a]	hydrophilic	moderately hydrophobic	hydrophobic
Acinetobacter haemolyticus AR-46[b]	hydrophilic	hydrophilic and hydrophobic	hydrophilic
Acinetobacter venetianus RAG-1[a]	hydrophobic	hydrophobic	hydrophobic
Rhodococcus sp. 094[c]	moderately hydrophobic	hydrophobic	hydrophilic
Dietzia sp. E1[d]	moderately hydrophobic	hydrophobic	hydrophilic

Data were taken from: [a] Baldi *et al.* (1999). [b] Bihari *et al.* (2007). [c] Bredholt *et al.* (2002). [d] This study.

harbour at least one *alkB* (FJ435354, FJ435355) and three CYP153 (FJ35357 – FJ35362) paralogs.

In order to detect such genes in *Dietzia* sp. E1 too, their amplifications in PCR reactions were achieved. Using the *alkB*-type integral membrane alkane hydroxylase sequence-specific TS2S/deg1RE degenerate primer pair, an amplicon with the expected length (557 bp) was obtained and cloned. The sequencing of ten independent clones revealed identical inserts. The internal *alkB* segment of isolate E1 displayed the highest similarity (89.16% and 88.75% identity) to that of *Dietzia* sp. H0B and *Dietzia* sp. H0, respectively.

Beside *alkB*, another partial alkane hydroxylase gene, the CYP153-type cytochrome P450, was successfully amplified using the P450fw1/P450rv3 degenerate primer pair. The PCR reaction yielded a 339-bp product, which was also cloned and sequenced. The partial *Dietzia* sp. E1 CYP153 gene sequences were found to be identical and exhibited the highest similarity (96% identity) to "UN" CYP153 clone (FJ435362) of *Dietzia* sp. H0B. The cytochrome P450 sequence of our isolate also shared relatively high, 83–89%, identity with the other five known *Dietzia* CYP153 homologues.

Discussion

A novel mesophilic and alkaliphilic strain isolated from a Hungarian petroleum-contaminated soil habitat was identified as *Dietzia* sp. E1. In contrast with most of the *Dietzia* spp., strain E1 was not halophilic or halotolerant. However, excellent *n*-alkane-degrading ability was observed and *n*-C_{12} to *n*-C_{38} alkane components of crude oil were utilized. As surveyed in the Introduction section, some other *Dietzia* spp. are also able to deplete a broad range of *n*-alkanes, but detailed analysis on substrate mineralization by these strains has not been reported at all. In the case of *Dietzia* sp. E1, the preferred substrate was not an easily accessible medium-chain liquid *n*-alkane, but the very long-chain *n*-C_{20} alkane as carbon source. During a 16-h incubation period, isolate E1 aerobically utilized 0.615 g/l *n*-eicosane as the sole carbon and energy source on MNPS minimal broth at 37 °C and pH 8. The total cell number increased from initial 10^6 cells/ml to final $9.8 \cdot 10^8$ cells/ml.

The high rate of *n*-alkane biodegradation coexisted with a special substrate adhesion mechanism, which was clearly distinguishable from that evolved in different members of the *Acinetobacter* genus, but was similar to that of *Rhodococcus* sp. 094 (summarized in Table I). Both *Dietzia* sp. E1 and *Rhodococcus* sp. 094 cells possess three distinct levels of cell-surface hydrophobicity during growth. Moderate hydrophobicity can be observed when cells grow on water-soluble substrates. After exposure to *n*-alkanes, it turns to extreme hydrophobicity in the exponential phase of growth, whilst cells suddenly become hydrophilic reaching the stationary phase. Whyte *et al.* (1999) and Bredholt *et al.* (2002) suggested that the release of extracellular polymeric substances and/or other hydrophobic factors can be responsible for the dramatic change of cell-surface hydrophobicity in *Rhodococcus* sp. Q15 and 094 strains, respectively. Considering that the rapid loss of hydrophobicity and the alkane growth substrate-binding ability coincided, the presence of such hydrophobic bioemulsifier is very likely in *Dietzia* sp. E1 too.

Once the substrate becomes bioavailable and penetrates the cell membrane, multi-step oxidation processes can occur. Based on the results of the GC-MS analysis, the *n*-alkane degradation continues when E1 cells enter the early stationary

phase and their surface hydrophobicity decreases. We could identify intracellular *n*-hexadecanal, an intermediate of the monoterminal hydroxylation pathway. Its content reached 45% of the amount of the intracellular *n*-hexadecane substrate. *n*-Hexadecanol was not detected in the cell extracts indicating that the activity of alkane hydroxylases and alcohol dehydrogenases may be much higher than that of aldehyde dehydrogenases. In order to seek terminal alkane hydroxylase sequences in the genome of strain E1, their PCR-based detections were achieved. A partial *alkB*-encoded integral membrane alkane hydroxylase and a partial CYP153-type cytochrome P450 alkane hydroxylase sequence were also identified, both of them shared high similarity with corresponding sequences of other *Dietzia* spp. AlkBs and CYPs are able to catalyze the same terminal alkane hydroxylation reaction, and can be present in the same organism with an overlapping substrate range (van Beilen *et al.*, 2006). Beside some *Mycobacterium* and *Rhodococcus* spp., strains of another Gram-positive actinomycete genus, *Dietzia* spp. seem to possess multiple alkane hydroxylases. This phenomenon can be physiologically advantageous and may explain the excellent *n*-alkane biodegradation rate and unusual substrate preference found in our novel isolate E1.

To the best of our knowledge, this is the first detailed analysis as concerns the effects of environmental parameters on hydrocarbon biodegradation rates, the alkane substrate preference, the alkane adhesion mechanism, and the catabolic pathway in this unexplored genus, which may support further investigations of other relevant *n*-alkane-degrading *Dietzia* isolates.

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