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Dietzia psychralcaliphila sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons

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A novel, facultatively psychrophilic alkaliphile that grows on a chemically defined medium containing *n*-alkanes as the sole carbon source was isolated from a drain of a fish product-processing plant. The isolate was an aerobic, non-motile, Gram-positive bacterium. The bacterium was catalase-positive and oxidase-negative. The cell wall contained *meso*-diaminopimelic acid, arabinose and galactose; the glycan moiety of the cell wall contained acetyl residues. The G+C content of the DNA was 69·6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate was closely related to members of the genus *Dietzia* (96·1–96·8% similarity). Comparisons of phenotypic and chemotaxonomic characteristics between the isolate and the two known *Dietzia* species showed that they were very similar. However, the isolate differed from the two known *Dietzia* species in growth temperature range and certain physiological characteristics. DNA–DNA hybridization revealed that the isolate had 38·4 and 49·7% relatedness, respectively, to *Dietzia maris* and *Dietzia natronolimnaea*. On the basis of the physiological and biochemical characteristics, the phylogenetic position as determined by 16S rRNA gene analysis and DNA–DNA relatedness, it is concluded that the isolate should be designated as a novel species, for which the name *Dietzia psychralcaliphila* sp. nov. is proposed. The type strain is ILA-1^T (= JCM 10987^T = IAM14896^T = NCIMB 13777^T).

Keywords: *Dietzia psychralcaliphila*, facultatively psychrophilic, alkaliphilic, *n*-alkanes, 16S rRNA phylogeny

INTRODUCTION

In the light of the growing concern over the Earth's environmental problems, bioremediation using micro-organisms shows great promise. Oil pollution of the soil or water is widespread. Once an oil spill occurs, it has a great negative impact on the entire surrounding area. To date, there have been few examples of bioremediation of oil-contaminated soils and water under psychrophilic conditions compared with examples under mesophilic conditions. Although degradation of oil is difficult at moderate temperatures, it is even more difficult at low temperatures. At low temperatures, the viscosity of oil increases, preventing the spread of the oil in soil and water. In addition, low temperatures prevent the volatilization of short-chain alkanes (less than C_{10}), thus increasing their solubility in the aqueous phase and their toxicity, which can delay microbial degradation. Long-chain alkanes are in a solid state at low temperatures. They can also delay the microbial degradation of oil. Cold-adapted micro-organisms capable of degrading oil hydrocarbons at low temperatures have been reported (Westlake *et al*., 1978; Whyte *et al*., 1996, 1998, 1999; MacCormack & Fraile, 1997; Margesin & Schinner, 1997a, b, c, 1999; Foght *et al*., 1999). However, few cold-adapted micro-organisms capable of degrading oil hydrocarbons have been identified to the species level to date.

In the present study, we isolated a cold-adapted alkaliphilic micro-organism that utilizes petroleum

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ILA-1^T is AB049630.

hydrocarbons over a wide pH range. We performed phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequences and found that the strain should be classified as a novel species belonging to the genus *Dietzia*. To the best of our knowledge, the isolate is the first reported facultatively psychrophilic alkaliphile that grows on a chemically defined medium containing *n*-alkanes as the sole carbon source.

METHODS

Bacterial strains and cultivation. A bacterial strain was isolated from water (6 °C, pH 7) obtained from a drain pool of a fish-egg-processing plant using a synthetic medium (AT medium) that consisted of 5 g KNO_3, 0·5 g KH_2PO_4, 0·5 g $MgSO_4 . 7H_2O$, 0·01 g $FeSO_4 . 7H_2O$, 0·02 g $CaCl_2 . 2H_2O$, 0·001 g $MnSO_4 . nH_2O$, 0·0005 g $ZnSO_4 . 7H_2O$ and 15 g agar in 1l100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 10) in deionized water, supplemented with vaporized *n*-tetradecane as the sole carbon source. *n*-Tetradecane was vaporized onto the surface of the agar plate by inverting the plate over a piece of filtration paper that had been soaked in *n*-tetradecane. After 1 month of aerobic incubation at 4 °C, strain ILA-1^T was isolated. In addition to this isolate, *Dietzia maris* JCM 6166^T and *Dietzia natronolimnaea* (originally named '*Dietzia natronolimnaios*') 15LN1^T were used as reference strains in determining DNA–DNA relatedness. The micro-organisms were cultivated using R broth (pH 7·2), consisting of 10 g bacto peptone (Difco), 5 g bacto yeast extract (Difco), 5 g bacto malt extract (Difco), 5 g bacto Casamino acids (Difco), 2 g bacto beef extract, 2 g glycerol, 50 mg Tween 80 and 1 g $MgSO_4 . 7H_2O$ in 1 l deionized water, with shaking (130 r.p.m.) until the late exponential phase of growth at 27 °C. Cells for analysis of fatty acids were prepared by using AT medium with 1% acetic acid and phosphate buffer (pH 7) instead of *n*-tetradecane and 100 mM $NaHCO_3/Na_2CO_3$ buffer. Other culture conditions were as described above.

Phenotypic characterization. For identification of the isolate, R broth was used as a basal medium. Cultures were incubated at 27 °C and characterized by the methods of Yamada & Komagata (1972) and Barrow & Feltham (1993) unless stated otherwise. Utilization of carbohydrates (1%, w/v), organic acids (1%, w/v), amino acids (0·5%, w/v) and hydrocarbons (1%, v/v) was tested using AT medium without *n*-tetradecane and with phosphate buffer (pH 7) instead of 100 mM $NaHCO_3/Na_2CO_3$ buffer. Hydrolysis of lipids was estimated using a medium containing 5 g polypeptone (Nihon Pharmaceutical), 3 g yeast extract (Kyokuto), 10 ml tributyrin and 15 g agar in 1 l deionized water. When hydrocarbons were not used as the substrate, cultures were incubated at 27 °C for 2 weeks. Utilization of hydrocarbons was estimated at 5 °C for 2 months. Tributyrin was sterilized separately and emulsified in the medium. The requirement for and tolerance of NaCl were determined using a medium containing 1 g bacto peptone, 0·1 g yeast extract and 0–200 g NaCl in 1 l deionized water (pH 7·5).

Electron microscopy. For observation of negatively stained cells under a transmission electron microscope (TEM), cells were grown on R agar (R broth supplemented with 2% agar) for 3 d, after which the cells were suspended in physiological saline solution. A small drop of the suspension was placed on a carbon-coated copper grid and the cells were negatively stained with 1% (w/v) phosphotungstic acid and observed under a TEM (model H-800; Hitachi). For the scanning electron microscope (SEM), cells were grown on R agar and were immersed in a 2% (v/v) glutaraldehyde solution in 0·1 M cacodylate buffer (pH 7·0) for 2 h. After washing three times with 0·1 M cacodylate buffer, cells were fixed in 1% (w/v) OsO_4 for 2 h, dehydrated in a graduated ethanol series (50–100%, v/v) and substituted with amyl acetate. Preparations were dried to a critical point in CO_2, fixed on a specimen mount and sputter-coated with platinum and palladium. The specimen were observed under an SEM (model S-4000; Hitachi) at 3·0 kV.

Chemotaxonomic characterization. Analyses of cellular fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 1998). Trimethylsilylated derivatives of mycolic acids were analysed as described by Rainey *et al.* (1995b). The *meso*-diaminopimelic acid in the cell wall was identified by TLC (art. no. 5552, DC-Alufoline cellulose; Merck) as described by Yamada & Komagata (1970). The glycolate test was performed based on the method of Uchida & Aida (1977).

DNA base composition and DNA–DNA hybridization. Bacterial DNA was prepared according to the method of Marmur (1961). The DNA obtained was digested with nuclease P1 (Yamasa Shoyu) and the resulting nucleotides were separated by HPLC (Tamaoka & Komagata, 1984). The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and black microplates.

16S rRNA gene sequencing. The 16S rRNA gene sequence corresponding to positions 27–1519 in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978) was amplified by PCR. The 1·5-kb PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 377; Applied Biosystems). Multiple alignments of the sequence were performed and the nucleotide substitution rate (K_{nuc} value) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson *et al.*, 1994). Similarity values for sequences were calculated using the GENETYX computer program (Software Development).

RESULTS AND DISCUSSION

Morphology

Colonies of strain ILA-1^T on R agar were circular, convex, entire, opaque and coral red. Cells were Gram-positive, non-motile, non-spore-forming rods, 0·8–1·0 by 1·0–2·2 μm in size (Fig. 1). Cells exhibited a snapping-type division.

Phenotypic characteristics

Strain ILA-1^T exhibited the following phenotypic characteristics. Catalase and oxidase reactions were positive. The strain was negative for reduction of nitrate, H_2S production, urease, indole production, the Voges–Proskauer test and methyl red. Growth occurred between pH 7 and 10; the optimum pH was pH 9–10 in R broth. The strain grew in media supplemented with 0–10% NaCl but not in media with more than 12·5% NaCl. It grew at 5–30 °C, but not at 40 °C or higher. The isolate hydrolysed lipid and Tweens 20, 40, 60 and 80, but not casein, gelatin, starch or DNA. It utilized D-glucose, pyruvate, acetate, *n*-butyrate, isobutyrate and ethanol but not D-xylose,

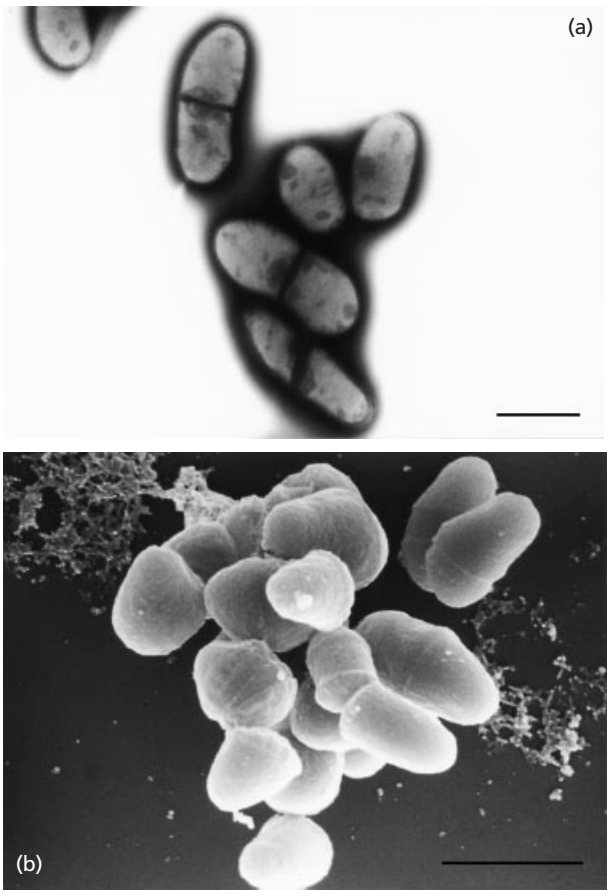

Fig. 1. (a) Transmission electron micrograph of negatively stained cells of *Dietzia psychralcaliphila* ILA-1^T. The cells were grown on R agar for 48 h at 27 °C. (b) Scanning electron micrograph of cells of platinum/palladium-coated *Dietzia psychralcaliphila* ILA-1^T. Bars, 1 μm.

D-mannose, raffinose, trehalose, D-cellobiose, lactose, gluconate, glucuronate, α-D-galacturonate, glutarate, DL-malate, glutamate, α-ketoglutarate, fumarate, DL-glycerate, L-tartrate, L-aspartate, arabitol, aconitate, *myo*-erythritol, D-glycerol, *p*-hydroxybenzoate, D-α-alanine, L-leucine, L-tyrosine, L-lysine, L-arginine, L-ornithine or DL-phenylalanine. Other characteristics are described in Table 1. Utilization of hydrocarbons by strain ILA-1^T was also tested using AT medium (pH 7) at 5 °C. It utilized *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, *n*-eicosane, *n*-tetracosane, *n*-octacosane and pristane but not *n*-dodecane, *n*-dotriacontane, cyclododecane, fluorene, anthracene or pyrene. Strain ILA-1^T utilized *n*-tetradecane over a wide pH range (6–10) and the optimum pH was 10 at 27 °C. The strain is expected to have potential for *in situ* bioremediation of oil-contaminated soil and water at low temperatures over a wide pH range.

Chemotaxonomic characteristics

GC analysis of methyl ester derivatives of cellular fatty acids of the strain revealed that the major components were $C_{16:0}$ (25%), $C_{16:1}$ (18%), 10-Me$C_{18:0}$ (22%) and $C_{18:1}$ (25%) and the minor components were $C_{13:0}$ (2%), $C_{17:1}$ (2%) and $C_{18:0}$ (2%). The amount of total unsaturated fatty acids was 45·2%. The cell wall of the strain contained *meso*-diaminopimelic acid, arabinose and galactose. Other characteristics are described in Table 2.

Table 1. Comparison of phenotypic characteristics of *Dietzia* species

Taxa are identified as: 1, *D. psychralcaliphila*; 2, *D. maris*; 3, *D. natronolimnaea*. Culture was performed under neutral conditions. +, Positive; −, negative; NA, not available. All strains were able to grow at pH 10 and 7 on R medium. All strains were positive for utilization of acetate, D-fructose, D-glucose, propionate, 3-hydroxybutyrate and valerate and negative for utilization of D-galactose, lactose, sucrose, salicin, D-melibiose, D-sorbitol, L-arabinose, caprate, histidine, rhamnose, D-ribose, inositol, maltose, DL-lactate, L-alanine and *N*-acetylglucosamine.

Characteristic	1	2*	3†
Utilization of:			
Glutamate	−	+	+
Succinate	−	−	+
Mannitol	−	−	+
Citrate	−	−	+
L-Proline	−	−	+
Reduction of nitrate	−	+	NA
H_2S production	−	+	NA
Urease	−	+	NA

* Data for utilization of substrates were obtained in this study using AT medium.

† Data were obtained from Duckworth *et al.* (1998).

Table 2. Comparison of chemotaxonomic characteristics of *Dietzia* species

Taxa are identified as: 1, *D. psychralcaliphila*; 2, *D. maris*; 3, *D. natronolimnaea*. Data for *D. maris* were obtained in this study. All three taxa contain straight-chain saturated, monounsaturated and 10-methyl branched fatty acids, *meso*-diaminopimelic acid as the major peptidoglycan diamino acid and MK-8(H_2) as the major menaquinone. NA, Not available.

Character	1	2	3
N-Glycolyl in glycan moiety of cell wall	−	−	NA
DNA G+C content (mol%)	69·6	70·4	66·1
Mycolic acid chain length	34–39	33–38	34–38

16S rRNA gene sequence analysis

The nucleotide sequence of the 16S rRNA gene amplified enzymically from strain ILA-1^T was determined by direct automated sequencing. A total of

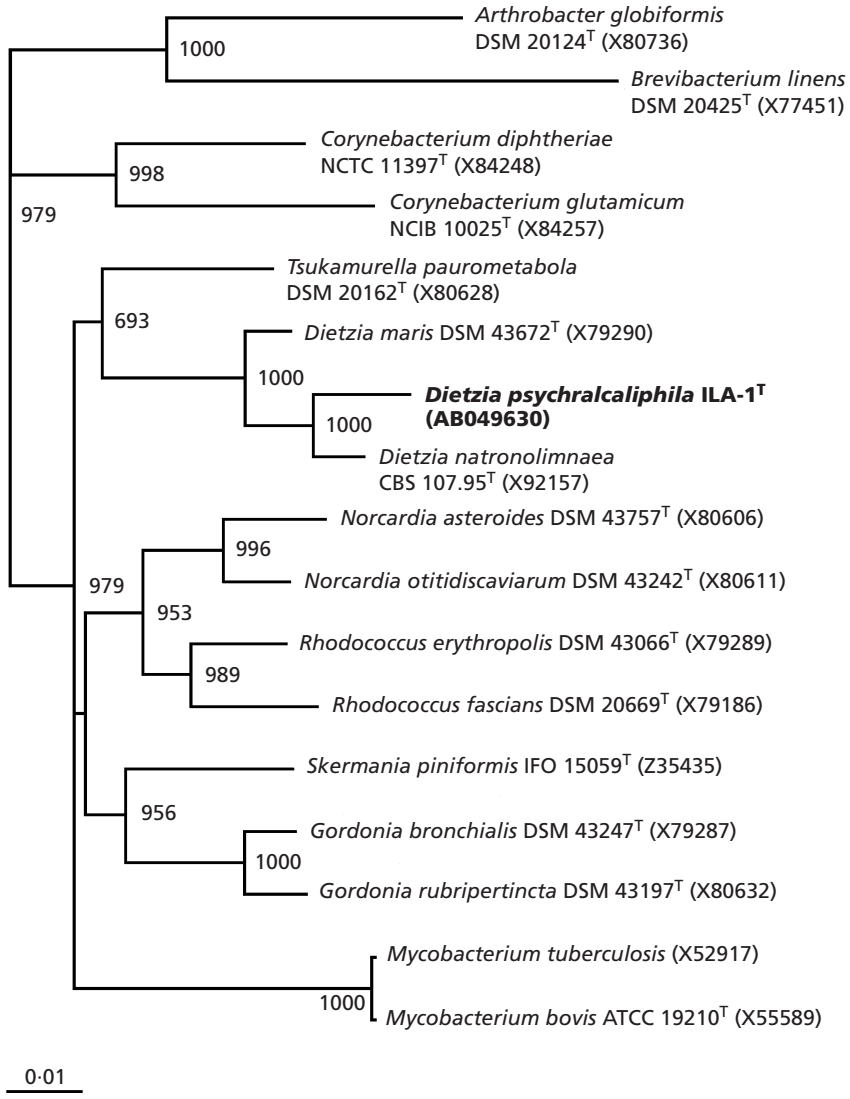

Fig. 2. Phylogenetic tree of *Dietzia psychralcaliphila* ILA-1^T and reference organisms derived from 16S rRNA gene sequence data using the neighbour-joining method. Numbers indicate bootstrap values greater than 500. Bar, 0·01 K_{nuc} unit.

1483 nucleotides was sequenced and the primary structure was aligned with those of 16 representative members of wall chemotype IV (taxa containing *meso*-diaminopimelic acid, arabinose and galactose; Lechevalier & Lechevalier, 1970), which contain mycolic acids, and other related organisms. A phylogenetic tree was constructed on the basis of the distance matrix data (Fig. 2). It showed that strain ILA-1^T formed a coherent cluster with species of the genus *Dietzia*. The genus contained only two species, *D. maris* and *D. natronolimnaea*. The degrees of sequence similarity of strain ILA-1^T to *D. maris* and *D. natronolimnaea* were respectively 96·1 and 96·8 %.

DNA base composition and DNA–DNA hybridization

The DNA G+C content of strain ILA-1^T was 69·6 mol %. According to the results of 16S rRNA gene sequence analysis, strain ILA-1^T is included in the genus *Dietzia*. The levels of DNA–DNA relatedness of strain ILA-1^T to *D. maris* and *D. natronolimnaea* were determined to be 38·4 and 49·7 %, respectively.

Distribution of *Dietzia* strains

Dietzia strains have been isolated from soil, skin and intestinal tracts of the carp (Nesterenko *et al.*, 1982; Rainey *et al.*, 1995b), deep-sea sediments (Colquhoun *et al.*, 1998), the deepest sea mud of the Mariana Trench (Takami *et al.*, 1997) and soda lakes (Jones *et al.*, 1998; Duckworth *et al.*, 1998). Furthermore, *Dietzia* strains are able to grow at alkaline as well as neutral pH. These results suggest that *Dietzia* strains are distributed widely in nature and a still wider distribution is expected. In the present study, strain ILA-1^T was isolated from a drain pool of a fish-egg-processing plant. Several kinds of marine fish are processed in this plant. This suggests that *Dietzia* strains may exist in the skin or intestinal tracts of marine fish. However, further studies are needed to clarify this.

Conclusions

Although strain ILA-1^T did not show significantly high levels of DNA–DNA relatedness to *D. maris* and *D. natronolimnaea*, the strain was difficult to discriminate from the two species of the genus *Dietzia* on the basis of physiological and biochemical characteristics (Table 1) and chemotaxonomic properties (Table 2). This observation is in accordance with a report that physiological and biochemical characteristics and chemotaxonomic properties are not particularly discriminatory for mycolic acid-containing bacteria (Rainey *et al.*, 1995a). Based on physiological and biochemical characteristics, the phylogenetic position as determined by 16S rRNA gene analysis and DNA–DNA relatedness, the name *Dietzia psychralcaliphila* sp. nov. is proposed for this novel organism.

Description of *Dietzia psychralcaliphila* sp. nov.

Dietzia psychralcaliphila (psy.chral.ca.li.phil′a. Gr. adj. *psychros* cold; N.L. *alcali* alkali, from Arabic *al qali* potash soil; Gr. adj. *philos* friendly to; N.L. fem. adj. *psychralcaliphila* loving cold, alkaline environments).

Cells are Gram-positive, non-motile, non-spore-forming rods (0·8–1·0 by 1·0–2·2 μm). Cells show a snapping-type division. Colonies are circular, convex, glistening and coral red. Catalase and oxidase reactions are positive. Negative for reduction of nitrate, H_2S production, urease, indole production, the Voges–Proskauer test and methyl red. Growth occurs between pH 7 and 10 in R broth. Grows in media supplemented with 0–10 % NaCl but not in media with salinity higher than 12·5 %. Grows at 5–30 °C, but not at 40 °C or higher. The major isoprenoid quinone is MK-8(H_2). The whole-cell fatty acids consist of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and 10-Me$C_{18:0}$ as tuberculostearic acid. Short-chain mycolic acids are present (34–39 carbon atoms). The cell wall contains *meso*-diaminopimelic acid, arabinose and galactose; the glycan moiety of the cell wall contains acetyl residues. The isolate hydrolyses lipid and Tweens 20, 40, 60 and 80, but not casein, gelatin, starch or DNA. Utilizes D-glucose, D-fructose, propionate, valerate, 3-hydroxybutyrate, pyruvate, acetate, *n*-butyrate, isobutyrate, ethanol, *n*-tridecane, *n*-pentadecane, *n*-hexadecane, *n*-eicosane, *n*-tetracosane and pristane but not D-xylose, D-arabinose, D-mannose, D-galactose, raffinose, sucrose, trehalose, D-cellobiose, melibiose, lactose, maltose, D-ribose, rhamnose, salicin, gluconate, glucuronate, α-D-galacturonate, glutarate, DL-malate, DL-lactate, citrate, glutamate, α-ketoglutarate, succinate, fumarate, DL-glycerate, caprate, L-tartrate, L-aspartate, arabitol, aconitate, mannitol, D-sorbitol, inositol, *myo*-erythritol, D-glycerol, *p*-hydroxybenzoate, L-α-alanine, D-α-alanine, L-leucine, histidine, L-proline, L-tyrosine, L-lysine, L-arginine, L-ornithine, DL-phenylalanine, *N*-acetylglucosamine, *n*-decane, *n*-dotriacontane, cyclododecane, fluorene, anthracene or pyrene. The DNA G+C content is 69·6 mol % (determined by HPLC).

The type strain, ILA-1^T, has been deposited at The Institute of Physical and Chemical Research (RIKEN), Wako, Japan, as JCM 10987^T, at the IAM Culture Collection, The University of Tokyo, Tokyo, Japan, as IAM 14896^T and at the National Collection of Industrial and Marine Bacteria, Aberdeen, UK, as NCIMB 13777^T.

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