Brachybacterium phenoliresistens sp. nov., isolated from oil-contaminated coastal sand

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A coccoid- to ovoid-shaped, Gram-positive, non-motile bacterial strain, designated phenol-A^T, was isolated from an oil-contaminated coastal sand sample collected from Pingtung County, southern Taiwan, and characterized by use of a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequences showed that the novel strain formed a monophyletic branch at the periphery of the evolutionary radiation occupied by the genus *Brachybacterium* in the family *Dermabacteraceae*, class *Actinobacteria*. The closest neighbours were *Brachybacterium rhamnosum* LMG 19848^T (96.9% 16S rRNA gene sequence similarity), *Brachybacterium nesterenkovi* DSM 9573^T (97.0%) and *Brachybacterium muris* C3H-21^T (96.3%). The peptidoglycan type of strain phenol-A^T was variation A4 γ with meso-diaminopimelic acid as the diagnostic cell-wall diamino acid. The isolate contained MK-7 as the major component of the quinone system. The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylglycerol, and unidentified phospholipids and glycolipids. The predominant fatty acid was anteiso-C_{15:0} (59.5%); significant amounts of iso-C_{16:0} (9.4%), iso-C_{14:0} (9.5%) and anteiso-C_{17:0} (10.8%) were also present. The isolate was also distinguished from recognized members of the genus *Brachybacterium* on the basis of several phenotypic and biochemical characteristics. It is evident from the genotypic, chemotaxonomic and phenotypic data that isolate phenol-A^T represents a novel species of the genus *Brachybacterium*, for which the name *Brachybacterium phenoliresistens* sp. nov. is proposed. The type strain is phenol-A^T (=LMG 23707^T=BCRC 17589^T).

The genus *Brachybacterium*, first proposed by Collins *et al.* (1988), belongs to the family *Dermabacteraceae*, class *Actinobacteria*. At the time of writing, the genus *Brachybacterium* encompasses ten recognized species: *Brachybacterium alimentarium*, *B. conglomeratum*, *B. faecium*, *B. fresconis*, *B. muris*, *B. nesterenkovi*, *B. paraconglomeratum*, *B. rhamnosum*, *B. sacelli* and *B. tyrofermentans*. The aim of the present study was to determine the taxonomic position of strain phenol-A^T, which was isolated from an oil-contaminated coastal sand sample.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of phenol-A^T is DQ822566.

A figure showing the polar lipid profile of strain phenol-A^T and a table detailing the fatty-acid profile of strain phenol-A^T in comparison with recognized members of the genus *Brachybacterium* are available as supplementary material with the online version of this paper.

A phenol-resistant bacterial strain, designated phenol-A^T, was isolated on nutrient agar (BD Difco) supplemented with 3% (w/v) NaCl and 0.05% (w/v) phenol from a contaminated coastal sand sample collected from Pingtung County, Taiwan. The organism was maintained and subcultivated further on marine agar 2216 (BD Difco) or in marine broth 2216 (BD Difco). The novel strain was preserved at –80 °C in a 20% (v/v) glycerol suspension in marine broth 2216 or by lyophilization.

The bacterial cells were observed via phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth to ascertain their morphology. Motility of cells was tested by use of a hanging-drop method. Flagellum staining was performed by using Spot Test Flagella Stain (BD Difco). The Gram Stain Set (BD Difco) and Ryu non-staining KOH method (Powers, 1995)

were used to ascertain the Gram reaction. Accumulation of poly- β -hydroxybutyrate granules was tested via light microscopy after staining cells with Sudan black. Colony morphology was studied by using a stereoscopic microscope (SMZ 800; Nikon). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992) and Bernardet *et al.* (2002). The pH range for growth was examined in marine broth 2216 medium by using appropriate biological buffers (pH 4–10 at intervals of 1.0 pH units) (Chung *et al.*, 1995). Tolerance to various levels of NaCl was tested in nutrient broth prepared according to the formula of the Difco medium, but the NaCl concentrations were altered as required (0, 0.5 and 1.0–10 %, w/v, at intervals of 1.0 %). Growth at various temperatures (4–45 °C) was measured on marine broth 2216. Growth was examined by measuring the turbidity (OD₆₀₀) of cultures grown over various pH, NaCl and temperature ranges. Anaerobic cultivation was performed on marine agar 2216 by using an Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). The 16S rRNA gene sequence of strain phenol-A^T was obtained by using a DNA sequencer (ABI PRISM 310; Applied Biosystems) and the sequence was then assembled by using the Fragment Assembly System program in the Wisconsin Package 9.1 (GCG, 1995). A nearly complete 16S rRNA gene sequence (1457 nt) of strain phenol-A^T was compared with 16S rRNA gene sequences available from the RDP, GenBank and EzTaxon (Chun *et al.*, 2007) databases. Multiple sequence alignments including strain phenol-A^T and its closest relatives were performed by using BioEdit software (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony

(Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm according to the Jukes & Cantor (1969) distance model, and bootstrap analysis for the neighbour-joining tree was performed based on 1000 resamplings. A comparison of the 16S rRNA gene sequence of strain phenol-A^T with those of members of the genera classified in the family *Dermabacteraceae*, class *Actinobacteria*, showed that the novel strain fell within the evolutionary radiation occupied by the genus *Brachybacterium* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain phenol-A^T formed a coherent cluster with *B. nesterenkovi* DSM 9573^T, *B. rhamnosum* LMG 19848^T and *B. muris* C3H-21^T, but its position within the genus was not particularly clear as the branching order between many species of the genus was not supported by bootstrap values above 50 %. Similar tree topologies were obtained in phylogenetic trees generated with the maximum-parsimony and maximum-likelihood algorithms (data not shown). Based on 16S rRNA gene sequence similarity calculations, strain phenol-A^T was most closely related to *B. sacelli* LMG 20345^T (97.6 %), *B. nesterenkovi* DSM 9573^T (97.0 %), *B. rhamnosum* LMG 19848^T (96.9 %), *B. paraconglomeratum* LMG 19861^T (96.8 %), *B. fresconis* LMG 20336^T (96.3 %), *B. faecium* DSM 4810^T (96.5 %), *B. alimentarium* CNRZ 925^T (96.4 %), *B. conglomeratum* NCIB 9859 (96.4 %), *B. muris* C3H-21^T (96.3 %) and *B. tyrofermentans* CNRZ 926^T (96.3 %). The level of gene sequence similarity between strain phenol-A^T and *Dermabacter hominis* DSM 7083^T, a species from another genus within the family *Dermabacteraceae*, was only 95.1 %.

Whole genome DNA–DNA hybridization experiments were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The level of hybridization

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the European Molecular Biology Laboratory database (accession numbers are given in parentheses) constructed after multiple alignments of data showing the position of strain phenol-A^T within the genus *Brachybacterium*. Distance calculations and clustering with the neighbour-joining method were performed by using the software package BioEdit. Numbers at nodes are percentage bootstrap values based on 1000 resampled data sets; only values above 50 % are given. Bar, 1 % sequence dissimilarity per nucleotide position.

was calculated by means of triplicate experiments. The separate species status of strain phenol-A^T was demonstrated by the hybridization values obtained when it was hybridized with *B. rhamnosum* DSM 10240^T, *B. nesterenkovi* DSM 9573^T and *B. muris* DSM 15460^T, which showed only 20.6 ± 3.1 , 15.0 ± 3.6 and 11.2 ± 2.7 % DNA–DNA relatedness to strain phenol-A^T, respectively. These DNA–DNA relatedness values were significantly lower than that accepted as demarcating species (Wayne *et al.*, 1987). Although strain phenol-A^T showed highest 16S rRNA gene sequence similarity to *B. sacelli* LMG 20345^T (97.6 %), DNA–DNA hybridization was not carried out because strain phenol-A^T formed a separate subclade in the phylogenetic tree with a bootstrap value <50 % (Fig. 1). Based on the above DNA–DNA relatedness data, strain

phenol-A^T warrants description as representing a novel species in the genus *Brachybacterium*.

The DNA G + C content of strain phenol-A^T was estimated from duplicate experiments as described by Mesbah *et al.* (1989). The nucleoside mixture was separated with an HPLC system. The DNA G + C content of strain phenol-A^T was 70.8 (±0.5) mol%, consistent with previously reported values for members of the genus *Brachybacterium* (68.6–73 mol%; Table 1).

Chemosystematic studies were carried out to establish whether strain phenol-A^T had a chemical profile consistent with its assignment to the genus *Brachybacterium*. Biomass for these studies was grown in marine agar 2216 for 3 days at 28 °C. Fatty acid methyl esters were prepared, separated

Table 1. Differential characteristics between strain phenol-A^T and recognized species of the genus *Brachybacterium*

Taxa: 1, strain phenol-A^T; 2, *B. alimentarium* CCM 4520^T; 3, *B. conglomeratum* CCM 2589^T; 4, *B. faecium* CCM 4372^T; 5, *B. fresconis* DSM 14564^T; 6, *B. muris* C3H-21^T; 7, *B. nesterenkovi* CM 2432; 8, *B. paraconglomeratum* DSM 46341^T; 9, *B. rhamnosum* DSM 10240^T; 10, *B. sacelli* DSM 14566^T; 11, *B. tyrofermentans* CCM 4521^T. Data for the reference species were obtained from Takeuchi *et al.* (1995), Schubert *et al.* (1996), Heyrman *et al.* (2002) and Buczolits *et al.* (2003). +, Positive; (+), weakly positive; –, negative; ND, not determined; F, facultative; A, aerobic; M, microaerophilic.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Source	Oil-polluted coastal sand	Cheese	Oil brine	Poultry deep litter	Mural paint-ings	Mouse	Cheese	Poultry deep litter	Corn steep liquor	Mural paint-ings	Cheese
Oxidase	–	+	–	–	–	–	–	–	–	–	+
Growth	F	F	F	A	A	M	F	F	F	A	F
Nitrate reduction	–	+	+	+	+	+	+	+	+	+	+
Urease production	–	+	+	–	+	–	–	+	+	–	–
H ₂ S production	–	–	+	–	–	–	+	+	+	–	–
Growth at 15 % NaCl	–	+	+	+	+	–	–	(+)	–	+	(+)
Hydrolysis of:											
Starch	+	+	+	+	–	+	–	+	+	–	–
Tween 20	+	+	–	–	–	+	+	–	–	–	–
Tween 80	–	–	–	–	–	(+)	–	–	–	–	–
Acid production from:											
D-Arabinose	+	(+)	–	(+)	–	+	–	–	–	–	–
DL-Lactose	(+)	–	–	–	–	+	+	(+)	–	–	–
Maltose	+	–	+	(+)	–	+	(+)	+	+	–	–
L-Rhamnose	+	+	–	–	(+)	+	–	–	–	(+)	(+)
D-Ribose	–	(+)	–	(+)	–	+	(+)	–	(+)	–	(+)
D-Sorbitol	–	–	–	–	–	–	–	–	+	–	–
Sucrose	+	–	–	–	–	+	+	+	+	–	–
D-Xylose	+	(+)	–	–	(+)	–	+	–	(+)	–	–
Peptidoglycan with:											
Glycine	+	+	+	+	+	+	–	+	+	+	+
Aspartic acid	+	+	–	–	–	+	–	–	–	–	+
DNA G + C content (mol%)	70.8	73.0	70.6	69.4	70.4	ND	71.2	68.6	71.5	70.3	73.0

and identified according to the instructions of the Microbial Identification System (MIDI; Microbial ID) (Sasser, 1990). Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Purified cell-wall preparations were obtained by the method of Schleifer & Kandler (1972) and the amino acids in peptidoglycan hydrolysates were analysed as described by Schleifer (1985). Menaquinones were extracted and analysed by HPLC as described by Collins (1994) along with those from *B. muris* DSM 15460^T, *B. nesterenkovi* DSM 9573^T and *B. rhamnosum* DSM 10240^T as reference strains. The predominant fatty acids in strain phenol-A^T were anteiso-C_{15:0} (59.5 %), anteiso-C_{17:0} (10.8 %), iso-C_{14:0} (9.5 %) and iso-C_{16:0} (9.4 %) (see Supplementary Table S1 in IJSEM Online). This fatty-acid profile is in good agreement with the major characteristics of members of the genus *Brachybacterium* (Collins *et al.*, 1988; Gvozdyak *et al.*, 1992; Takeuchi *et al.*, 1995; Schubert *et al.*, 1996; Heyrman *et al.*, 2002; Buczolits *et al.*, 2003). Strain phenol-A^T contained MK-7 as the major component of the quinone system, as found for other species of the genus *Brachybacterium* (Takeuchi *et al.*, 1995; Buczolits *et al.*, 2003). Diphosphatidylglycerol and phosphatidylglycerol were the predominant polar lipids. In addition, four unknown glycolipids and one unknown phospholipid were detected. These data are again similar to those for other members of the genus *Brachybacterium* (Collins *et al.*, 1988; Takeuchi *et al.*, 1995; Buczolits *et al.*, 2003) (see Supplementary Fig. S1 in IJSEM Online). The characteristic cell-wall amino acids for strain phenol-A^T were meso-diaminopimelic acid, alanine, glycine, aspartic acid and glutamic acid. The peptidoglycan type was variant A4 γ (Schleifer & Kandler, 1972), consistent with other members of the genus *Brachybacterium* (Gvozdyak *et al.*, 1992; Schubert *et al.*, 1996; Heyrman *et al.*, 2002; Buczolits *et al.*, 2003). However, phenol-A^T could be differentiated from *B. nesterenkovi* based on the presence of glycine and aspartic acid, and from *B. conglomeratum*, *B. faecium*, *B. fresconis*, *B. paraconglomeratum*, *B. rhamnosum* and *B. sacelli* based on the presence of aspartic acid (Table 1).

Strain phenol-A^T was examined for a broad range of phenotypic properties. Catalase, oxidase, DNase and lipase activity, motility, production of acid from various carbohydrates, and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined according to standard methods (Gerhardt *et al.*, 1994; Lanyi, 1987; MacFaddin, 2000). Additional biochemical tests were performed by using the API Coryne, API 20E, API ZYM (all bioMérieux) and Biolog GP2 (Biolog) microtest systems according to the methods outlined by the manufacturers. Sensitivity to antibiotics was examined after spreading cells (0.5 McFarland units) on marine 2216 agar plates. The antibiotic discs contained the following: ampicillin (10 μ g), chloramphenicol (30 μ g), erythromycin (15 μ g), gentamicin (10 μ g), kanamycin (30 μ g), nalidixic acid (30 μ g), novobiocin (30 μ g), rifampicin (5 μ g),

penicillin G (10 U), streptomycin (10 μ g) or tetracycline (30 μ g). The effect of antibiotics on cell growth was assessed after 3 days incubation and susceptibility was scored based on the distance from the edge of any clear zone to that of the disc. If the distance was greater than 3 mm, the strain was classified as susceptible, 1–3 mm, it was classified as moderately susceptible and if the clear zone was less than 1 mm, it was considered resistant. Detailed results of the biochemical characterization of strain phenol-A^T are provided in Table 1 and in the species description below. A comparison of the phenotypic characteristics of strain phenol-A^T with those of the type strains of related *Brachybacterium* species is presented in Table 1.

Based on 16S rRNA gene sequence comparisons, strain phenol-A^T occupies a separate position within the genus *Brachybacterium*. This is confirmed by its unique combination of chemotaxonomic characteristics (Table 1 and Supplementary Table S1) and biochemical traits (Table 1). It is clear from the genotypic and phenotypic data presented that strain phenol-A^T represents a novel species in the genus *Brachybacterium*, for which the name *Brachybacterium phenoliresistens* sp. nov. is proposed.

Description of *Brachybacterium phenoliresistens* sp. nov.

Brachybacterium phenoliresistens (phe.nol.i.res.ist.ens. N.L. neut. n. *phenol* phenol; L. part. adj. *resistens* resisting; N.L. part. adj. *phenoliresistens* phenol resisting, referring to the organism's ability to resist phenol).

Cells are small, coccoid to ovoid shaped, 0.8–1.3 μ m in diameter. Cells occur singly, in pairs or as agglomerates. Facultatively anaerobic, Gram-positive (Gram staining and KOH test), non-motile and non-spore-forming. Colonies on marine 2216 agar are pale yellow, circular and convex in shape with entire edges. Colonies are approximately 0.9–2.1 mm in diameter on marine 2216 agar after 48 h incubation at 28 °C. No accumulation of poly- β -hydroxybutyrate granules is observed. Flexirubin-type pigments are present. Optimum growth occurs aerobically, while only moderate anaerobic growth is observed. Good growth occurs at temperatures of 4–40 °C, 0.5–7.0 % NaCl and pH 5–10. Optimum growth occurs at 28–35 °C, 2–3 % NaCl and pH 7.0. Acid is produced from arabinose, fructose, mannose, raffinose, rhamnose, sucrose, xylose, cellobiose, galactose, glucose, lactose and trehalose, but not from ribose or sorbitol. Positive for catalase, DNase, and hydrolysis of starch and Tween 20. Weakly positive for hydrolysis of casein and Tween 40. Negative for oxidase, nitrate reduction, nitrite reduction, lipase (corn oil) and hydrolysis of Tweens 60 and 80. In the API Coryne system, positive for hydrolysis of pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -glucuronidase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase and aesculin and acid production from D-glucose,

D-xylose, D-mannitol, maltose, D-lactose and sucrose. Negative for nitrate reduction, urease, gelatin hydrolysis and acid production from D-ribose and glycogen. With the API 20E system, positive for ONPG test, acetoin production and acid production from glucose, mannitol, inositol, rhamnose, sucrose, amygdalin and arabinose, but negative for nitrate reduction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase, indole production, gelatinase and acid production from sorbitol and melibiose. Positive enzyme reactions (API ZYM) for alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase, weak positive reactions for C14 lipase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β -glucuronidase, but negative reactions for trypsin and α -chymotrypsin. The following carbon substrates (Biolog GP2) are oxidized: β -cyclodextrin, dextrin, glycogen, Tween 40, N-acetyl-D-glucosamine, L-arabinose, D-arabitol, arbutin, D-cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, methyl β -D-glucoside, palatinose, D-psicose, D-raffinose, salicin, D-sorbitol, stachyose, sucrose, trehalose, turanose, xylitol, pyruvic acid methyl ester, glycerol, adenosine and 2'-deoxyadenosine. Unable to oxidize α -cyclodextrin, inulin, mannan, Tween 80, N-acetyl- β -D-mannosamine, amygdalin, L-fucose, D-galacturonic acid, D-gluconic acid, myo-inositol, α -D-lactose, lactulose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl D-glucose, methyl α -D-glucoside, methyl α -D-mannoside, L-rhamnose, D-ribose, sedoheptulosan, D-tagatose, D-xylose, acetic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, inosine, thymidine, uridine, adenosine, uridine 5'-monophosphate, D-fructose 6-phosphate, α -D-glucose 1-phosphate, D-glucose 6-phosphate or DL- α -glycerol phosphate. Resistant to ampicillin, gentamicin, nalidixic acid and tetracycline, but sensitive to chloramphenicol, erythromycin, kanamycin, novobiocin, penicillin G, rifampicin and streptomycin. The diagnostic cell-wall diamino acid is *meso*-diaminopimelic acid and the peptidoglycan type is variation A4 γ containing amino acids *meso*-diaminopimelic acid, alanine, glycine, aspartic acid and glutamic acid. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, three unknown glycolipids and one unknown phospholipid. The predominant fatty acid is anteiso-C_{15:0} with significant amounts of iso-C_{16:0}, iso-C_{14:0} and anteiso-C_{17:0}. The major menaquinone is MK-7. DNA G + C content is 70.8 mol%.

The type strain, phenol-A^T (=LMG 23707^T=BCRC 17589^T), was isolated from oil-contaminated coastal sand, Pingtung County, Taiwan.

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