Deinococcus aquaticus sp. nov., isolated from fresh water, and Deinococcus caeni sp. nov., isolated from activated sludge

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The taxonomic positions of two environmental isolates from South Korea were established using a combination of genotypic and phenotypic data. The organisms, designated PB314T and Ho-08T, were Gram-negative, rod-shaped and non-sporo-forming and had chemotaxonomic properties consistent with their classification in the genus Deinococcus. 16S rRNA gene tree, the highest sequence similarities being shown to the type strains of Deinococcus grandis (96.3–96.7 %) and Deinococcus indicus (96.3–96.4 %). The isolates shared relatively high 16S rRNA gene sequence similarity (98.1 %) but had a DNA–DNA relatedness value of only 22 %. Chemotaxonomic data revealed that both strains possess quinone system MK-8 as the predominant compound, C16 : 1v7c and C16 : 0 as major fatty acids and ornithine as a diamino acid in the peptidoglycan structure, corroborating our assignment of the strains to the genus Deinococcus. The results of phylogenetic analyses based on 16S rRNA gene sequences, DNA–DNA relatedness values and physiological and biochemical tests clearly demonstrated that the two strains represent distinct species. On the basis of these data, two novel species, Deinococcus aquaticus sp. nov. (type strain PB314T = KCTC 12552T = NBRC 101311T) and Deinococcus caeni sp. nov. (type strain Ho-08T = KCTC 12553T = NBRC 101312T), are proposed.

The availability of improved taxonomic procedures facilitates the preliminary characterization of relatively large numbers of bacteria from environmental samples and hence the recognition of novel species. Over 100 putatively novel species were highlighted in our laboratory following the characterization of 300 aerobic phototrophic bacteria isolated from water and sediment samples collected either from the River Gapcheon, near Daejeon City, South Korea, or from a tributary of the river. One of the isolates, strain PB314, was tentatively assigned to the genus Deinococcus (unpublished results). A second strain, isolate Ho-08, was also considered to belong to the genus Deinococcus following the characterization of 128 bacterial strains isolated from activated sludge, compost and soil collected in the vicinity of Daejeon City in a second study designed to find novel bacterial species (Im et al., 2003).
The genus *Deinococcus* was established by Brooks & Murray (1981), and at present the genus comprises 27 species with validly published names. Although the 16S rRNA gene sequence similarity may be considered low enough to differentiate some species into other genera, the genus *Deinococcus* has not been divided because of the common characteristics of its members. Species of the genus *Deinococcus* are strictly aerobic, have high resistance to ionizing radiation, produce reddish, pink or yellow colonies, generally stain Gram-positive, have L-ornithine in the peptidoglycan and lack teichoic acids (Brooks & Murray, 1981; Ferreira et al., 1997; Rainey et al., 1997, 2005; Hirsch et al., 2004; Suresh et al., 2004; de Groot et al., 2005; Lai et al., 2006; Shashidhar & Bandekar, 2006; Weon et al., 2007). 16S rRNA gene sequence analyses show that the deinococci form a phylogenetically diverse group in a deeply branching lineage within the *Bacteria* (Rainey et al., 1997, 2005).

The aim of the present study was to determine the taxonomic positions of two environmental isolates using a polyphasic approach. The resultant data indicated that isolates PB314T and Ho-08T merit recognition as representatives of novel species of *Deinococcus*.

Strain PB314T was isolated from a freshwater stream of the River Gapcheon via direct plating onto 1:10-diluted R2A agar (Difco). Strain Ho-08T was isolated via direct plating onto R2A agar from an activated sludge in a wastewater-treatment plant that was constructed to treat the leachate flow from the landfill of Daejeon city. Colonies were subcultured from the isolation plates onto R2A agar plates and routinely cultured on R2A agar at 30 °C and maintained as glycerol suspensions (20 %, w/v) at −70 °C. Purified colonies were tentatively identified by using partial 16S rRNA gene sequences.

Genomic DNA was extracted and purified from each of the isolates using a commercial DNA extraction kit (Core Biosystem); PCR-mediated amplification of the 16S rRNA gene and sequencing of purified PCR products were carried out according to Kim et al. (2005). The resultant 16S rRNA gene sequences, compiled using SeqMan software (DNASTAR), were aligned using the BioEdit program (Hall, 1999), with corresponding sequences drawn from GenBank. Multiple alignments were performed by CLUSTAL_X program (Thompson et al., 1997) and gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA3 suite of programs (Kumar et al., 2004) and the maximum-likelihood method with the PHYLIP package (Felsenstein, 1989). Unrooted tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 replicates taken from the neighbour-joining database.

Nearly complete 16S rRNA gene sequences of strains PB314T and Ho-08T (1459 and 1458 bp, respectively) were obtained. Preliminary sequence comparison against 16S rRNA gene sequences deposited in GenBank indicated that our isolates belong to the genus *Deinococcus* of the class *Deinococci* (data not shown). On the basis of 16S rRNA gene sequence similarity, the closest cultured relatives were *Deinococcus grandis* DSM 3963T (96.3 and 96.7 %, respectively) and *Deinococcus indicus* Wt1aT (96.3 and 96.4 %, respectively) and the sequence similarity to the remaining *Deinococcus* type strains was less than 95 %. This relationship between our isolates and other members of the genus *Deinococcus* was also evident in the phylogenetic trees (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The sequences of the two strains showed high similarity (98.1 %), necessitating DNA–DNA hybridization tests.

The Gram reaction was performed using the non-staining method, as described by Buck (1982). Cell morphology and motility were observed under a Nikon light microscope at ×1000, with cells allowed to grow on R2A agar for 3 days at 30 °C. Catalase activity was determined by assessing bubble production in 3 % (v/v) H2O2; oxidase activity was determined using 1 % (w/v) tetrathymethyl-p-phenylenediamine. Substrate utilization as sole carbon sources and some physiological characteristics were determined using the API 32GN and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). Anaerobic growth was determined in serum bottles containing R2A broth supplemented with thioglycolate (1 g l−1) in which the upper air layer had been replaced with nitrogen. Nitrate and nitrite reduction were later confirmed by inoculating 25 ml serum bottles in triplicate, each containing 13 ml R2A broth, supplemented with KNO3 (10 mM) or NaNO2 (10 mM), respectively. Tests for degradation of DNA [in which DNase agar (Scharlau) plates were flooded with 1 M HCl], casein, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1993), and peptone (Atlas, 1993) were also used. The results are given in Table 1.

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolates Ho-08T and PB314T and representatives of the genus *Deinococcus*. Dots indicate phyletic lines that were also recovered using the maximum-parsimony algorithm. Numbers at nodes indicate percentages of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values above 70 % are given. Bar, 0.01 substitutions per nucleotide position.](image-url)
1987), xylan and cellulose (Ten et al., 2004) were performed and evaluated after 5 days. Growth at different temperatures (4, 15, 20, 25, 30, 37 and 42 °C) and various pH values (pH 5.0–10.0; at intervals of 0.5 pH units) was assessed after incubation for up to 5 days. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (TSA; Difco) and MacConkey agar was also evaluated, at 30 °C.

To determine the survival rate after exposure to gamma radiation, cultures were grown at 30 °C in liquid nutrient-rich medium TGY (1 % tryptone, 0.1 % glucose, 0.5 % yeast extract) or on TGY solid medium. To measure ionizing radiation resistance, cultures grown to the early stationary phase (~10^9 cells ml^-1) were divided into 1 ml aliquots without change of broth and were exposed on ice to a cobalt-60 gamma irradiator (point source; AECL, IR-79). The source strength was approximately 100 kCi at a dose rate of 70 Gy min^-1, and the actual doses were within 2 % of the target dose. Irradiated cells were diluted, plated in triplicate on TGY agar plates and incubated for 2 days before survivors were scored.

Colonies of strain PB314^Tcultured on R2A agar (Difco) at 30 °C were reddish, convex and circular. After 2 days incubation at 30 °C on R2A agar, colonies of strain Ho-08^T were pink and circular. Cells of both strains were aerobic, Gram-negative, non-motile rods. Strains PB314^T and Ho-08^T were able to grow at 20–37 °C, but did not grow at 4 or 42 °C. Growth at 30 °C was also observed on nutrient agar, but not on TSA or MacConkey agar. Physiological characteristics of strains PB314^T and Ho-08^T are summarized in the species descriptions and a comparison of selected characteristics with related type strains is shown in Table 1. Variation in the assimilation of various sole

<table>
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<td>l-Orn</td>
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<td>68.4</td>
<td>68.4–69.4</td>
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*Data from Oyaizu et al. (1987) and Suresh et al. (2004).
†Range of values for four strains.
carbon sources, in the hydrolysis of gelatin and in nitrate reduction was found between the two strains and their phylogenetically closest relatives of the genus *Deinococcus*. It is evident from the representative survival curves (Fig. 2) that the isolates are much more sensitive than the control strain to gamma radiation. Survival rates following exposure to 3 kGy were 7, 4 and 88 %, respectively, for isolates Ho-08T, PB314T and *Deinococcus radiodurans* R1. Isolate Ho-08T was approximately ten times more sensitive than strain PB314T to radiation doses of 9 and 15 kGy. Neither of the isolates demonstrated the characteristic shoulder of resistance in survival curves shown by highly resistant bacteria such as *D. radiodurans* (Battista et al., 1999). Radiation resistance is one of the main characteristics of the genus *Deinococcus*. For this reason, UV or gamma radiation has been used to isolate most organisms of the genus *Deinococcus* (Rainey et al., 2005; Oyaizu et al., 1987). However, a few novel species have been isolated from non-irradiated samples from air, soil, hot springs and culture contamination (Ferreira et al., 1997; Lai et al., 2006; Shashidhar & Bandekar, 2006; Weon et al., 2007). Isolation of strains Ho-08T and PB314T, with relatively low resistance to radiation, demonstrates that the extreme radiation resistance of the organisms is not a result of selection of resistant strains by irradiation but is a normal characteristic, as defined by Mattimore & Battista (1996). Significant difference in resistance to radiation strongly supports the suggestion that strains Ho-08T and PB314T were differentiated from other species within the genus *Deinococcus*.

Chemotaxonomic studies were carried out to establish whether the isolates had chemical markers that supported their assignment to the genus *Deinococcus*. Preparation of the cell wall and determination of peptidoglycan structure were carried out by the methods described by Schleifer & Kandler (1972) and cell-wall sugar analysis was carried out as described by Staneck & Roberts (1974). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified via TLC and subsequently analysed by HPLC, as described previously (Collins & Jones, 1981; Shin et al., 1996). For fatty acid methyl ester analysis, the strains were allowed to grow on R2A agar for 48 h at 30 °C and two loops of the well-grown cells were then harvested. Fatty acid methyl esters were prepared, separated and identified with the Sherlock Microbial Identification System (MIDI, Inc.) (Sasser, 1990). For measurement of the G+C content of the chromosomal DNA, genomic DNA was extracted and purified with the Qiagen Genomic-tip system 100/G and then degraded enzymically into nucleosides; the G+C content was determined as described by Mesbah et al. (1989) using reversed-phase HPLC.

Analysis of the peptidoglycan amino acids showed that strains PB314T and Ho-08T contained L-ornithine, alanine, glycine and glutamic acid. From the two-dimensional TLC patterns of peptides in partial peptidoglycan hydrolysates, strains PB314T and Ho-08T contained peptidoglycan type A3β (L-Orn–Gly–Gly). MK-8 was the predominant respiratory quinone of both strains PB314T and Ho-08T, like other *Deinococcus* species (Ferreira et al., 1997; Hirsch et al., 2004; Suresh et al., 2004; de Groot et al., 2005; Lai et al., 2006). They contained ribose and rhamnose in the cell wall. The predominant cellular fatty acids in the strains PB314T and Ho-08T were hexadecenoic acid (C16:1), which were also major fatty acids in most other *Deinococcus* species (Supplementary Table S1). However, some qualitative and quantitative differences in fatty acid composition could be observed between strains PB314T and Ho-08T and their phylogenetically closest relatives. In particular, in contrast to *D. grandis* DSM 3963T, *D. indicus* MTCC4913T and *D. radiodurans* ATCC 13939T, they produced C18:0 but not C15:1 and contained smaller amounts of C17:0. The fatty acid profile might be also useful for differentiation of the isolates. Strain Ho-08T differed from PB314T by the absence of C15:0, C15:0 iso, C17:0 iso, C15:1 and C16:1ω9c. The G+C contents of the genomic DNA of strains PB314T and Ho-08T were respectively 68.3 and 68.4 mol%. These properties are consistent with the assignment of the organisms to the genus *Deinococcus* (Brooks & Murray, 1981; Ferreira et al., 1997; Suresh et al., 2004; Lai et al., 2006).

The results of two-dimensional TLC analysis of polar lipids extracted from strains PB314T and Ho-08T are shown in Supplementary Fig. S2. Based on their staining behaviour, the polar lipid profiles of the two strains consisted of various unknown phosphoglycolipids, glycolipids and polar lipids; unknown phospholipids were also detected in strain Ho-08T. Polar lipid profiles of both strains were dominated by unknown phosphoglycolipid PGL2, which is consistent with previous results for *Deinococcus* species (Ferreira et al., 1997; de Groot et al., 2005; Lai et al., 2006; Weon et al., 2007). Moreover, strains PB314T and Ho-08T showed unknown phosphoglycolipid PGL1, glycolipids GL1 and
and GL₂ and unknown polar lipid L₁, which were also detected in other Deinococcus species (Lai et al., 2006; Weon et al., 2007). At the same time, each of the two strains showed unique combinations of unknown polar lipids that might be useful for distinguishing them from each other and other members of the genus Deinococcus.

DNA–DNA hybridization experiments were performed between PB31⁴ᵀ and Ho-08⁴ᵀ with the method described by Ezaki et al. (1989) using photobiotin-labelled DNA probes (Sigma) and microdilution wells (Greiner), with five replications for each sample. DNA–DNA relatedness of strain PB31⁴ᵀ to strain Ho-08⁴ᵀ was 22 %, which is low enough to differentiate strain PB31⁴ᵀ and strain Ho-08⁴ᵀ as members of different species (Wayne et al., 1987).

16S rRNA gene sequence comparison showed that strains PB31⁴ᵀ and Ho-08⁴ᵀ exhibited closest phylogenetic affinities to Deinococcus species. Phylogenetic analysis based on 16S rRNA gene sequences shows that the two strains fell within the radiation of the cluster comprising Deinococcus species and were most closely related to D. grandis DSM 3963⁷ᵀ, D. indicus Wi/1a⁷ᵀ and D. radiodurans DSM 20539⁷ᵀ. The presence of peptidoglycan type A3β and MK-8 as the major lipoquinone, the polar lipid patterns, fatty acid compositions and other characteristics confirm their assignment to the genus Deinococcus. Although strains PB31⁴ᵀ and Ho-08⁴ᵀ showed all the typical characteristics of the genus Deinococcus, they differed significantly from their closest phylogenetic relatives with respect to radiation resistance (Fig. 2), the combination of unknown polar lipids (Supplementary Fig. S2), the proportions of some fatty acids (Supplementary Table S1) and a number of other physiological and chemotaxonomic characteristics (Table 1). Furthermore, it is evident from the genotypic and phenotypic data that the two isolates could be clearly differentiated from each other. On the basis of these results, strains PB31⁴ᵀ and Ho-08⁴ᵀ are proposed as the type strains of two novel species of the genus Deinococcus, for which the names Deinococcus aquaticus sp. nov. and Deinococcus caeni sp. nov., respectively, are proposed.

Description of Deinococcus aquaticus sp. nov.

Deinococcus aquaticus (aqua’icus. L. masc. adj. aquaticus living, growing or found in or by water, aquatic).

Cells are Gram-negative, non-spore-forming rods, 2.0–3.0 μm long. Positive for catalase, β-galactosidase and hydrolysis of starch. Negative for urease, indole production, nitrate reduction and hydrolysis of DNA, CM-cellulose, lipid (olive oil) and xylan. Grows on R2A at pH 6.0–9.5. Does not require NaCl for growth; can tolerate 3 % (w/v) NaCl. The following substrates are utilized for growth as sole carbon sources: D-glucose, glycogen, maltose, N-acetyl-D-glucosamine, L-proline and sucrose. The following substrates are not utilized for growth: acetate, adipate, L-alanine, caprate, citrate, D-fucose, 4-hydroxybenzoate, 3-hydroxybenzoate, DL-3-hydroxybutyrate, inositol, itaconate, 2-ketogluconate, 5-ketogluconate, lactate, malonate, phenylacetate, propionate, L-rhamnose, D-ribose, L-serine, suberate and valerate. MK-8 is the predominant lipoquinone. The major fatty acids are C₁₆:1 and C₁₆:0. Ornithine and glycine are present in the peptidoglycan. Cell wall contains ribose and rhamnose. The G + C content of the genomic DNA of the type strain is 68.3 mol%.

The type strain, PB31⁴ᵀ (=KCTC 12552ᵀ =NBRC 101311ᵀ), was isolated from a freshwater stream that flows into the River Gapcheon near KAIST in Daejeon City, South Korea.

Description of Deinococcus caeni sp. nov.

Deinococcus caeni (cae’ni. L. gen. neut. n. caeni of sludge).

Cells are Gram-negative, non-spore-forming rods, 2.0–3.0 μm long. Positive for catalase, β-galactosidase and hydrolysis of starch. Negative for urease, oxidase, indole production, nitrate reduction and hydrolysis of DNA, CM-cellulose, lipid (olive oil) and xylan. Grows on R2A at pH 6.5–9.5. Does not require NaCl for growth; can tolerate 3 % (w/v) NaCl. The following substrates are utilized for growth as sole carbon sources: D-glucose, glycogen, maltose, N-acetyl-D-glucosamine, L-proline and sucrose. The following substrates are not utilized for growth: acetate, adipate, L-alanine, caprate, citrate, D-fucose, 4-hydroxybenzoate, 3-hydroxybenzoate, DL-3-hydroxybutyrate, inositol, itaconate, 2-ketogluconate, 5-ketogluconate, lactate, malonate, phenylacetate, propionate, L-rhamnose, D-ribose, L-serine, suberate and valerate. MK-8 is the predominant lipoquinone. The major fatty acids are C₁₆:1 and C₁₆:0. Ornithine and glycine are present in the peptidoglycan. Cell wall contains ribose and rhamnose. The G + C content of the genomic DNA of the type strain is 68.4 mol%.

The type strain, Ho-08⁴ᵀ (=KCTC 12553ᵀ =NBRC 101312ᵀ), was isolated from activated sludge in a wastewater-treatment plant that receives leachate from a landfill site in Daejeon City, South Korea.

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References


