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 PB314^T and Ho-08^T, were Gram-negative, rod-shaped and non-spore-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, C_{16:1}ω7c and C_{16:0} 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 PB314^T = KCTC 12552^T = NBRC 101311^T) and *Deinococcus caeni* sp. nov. (type strain Ho-08^T = KCTC 12553^T = NBRC 101312^T), 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 labor-

atory 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 One of the isolates, strain PB314, was tentatively assigned to the genus *Deinococcus* (unpublished results).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 GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PB314^T and Ho-08^T are respectively DQ017708 and DQ017709.

A 16S rRNA gene sequence-based maximum-likelihood tree, results of 2D TLC of polar lipids of strains PB314^T and Ho-08^T and a comparison of fatty acid profiles are available as supplementary material with the online version of this paper.

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 PB314^T and Ho-08^T merit recognition as representatives of novel species of *Deinococcus*.

Strain PB314^T was isolated from a freshwater stream of the River Gapcheon via direct plating onto 1:10-diluted R2A agar (Difco). Strain Ho-08^T 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 PB314^T and Ho-08^T (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 3963^T (96.3 and 96.7 %, respectively) and *Deinococcus indicus* Wt/1a^T (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) tetramethyl-*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,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolates Ho-08^T and PB314^T 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.

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 ($\sim 10^9$ 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^T cultured 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 1. Chemotaxonomic and phenotypic properties that separate isolates Ho-08^T and PB314^T from the type strains of phylogenetically close *Deinococcus* species

Strains: 1, PB314^T; 2, Ho-08^T; 3, *D. grandis* DSM 3963^T (unless indicated, data from Suresh *et al.*, 2004); 4, *D. indicus* MTCC4913^T (Suresh *et al.*, 2004; Weon *et al.*, 2007); 5, *D. radiodurans* ATCC 13939^T (Battista & Rainey, 2001; Rainey *et al.*, 2005). All strains are Gram-negative, non-motile, rod-shaped bacteria that produce catalase and utilize maltose, sucrose and L-proline as sole carbon sources. None of the strains hydrolyses urea or assimilates L-rhamnose, L-alanine, acetate or citrate as sole carbon sources. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Colony colour	Red	Pink	Red	Red	Pink–red
Temperature growth range (°C)	20–37	20–37	<30–42	20–37	>20 to <45
Tolerance to NaCl (% w/v)	0–3	0–3	0–1	0–1	ND
Oxidase	+	–	+	+	ND
β-Galactosidase	+	+	+	+	–
Nitrate reduction	–	–	+	+	+
Arginine dihydrolase	–	–	–	+	ND
Aesculin hydrolysis	+	–	+	+	–
Gelatin hydrolysis	–	–	+	+	ND
Utilization as carbon source of:					
L-Arabinose	–	–	–	+	–
Melibiose	+	–	–	+	ND
D-Glucose	+	+	+	–	+
D-Mannose	+	–	–	+	+
D-Ribose	–	–	–	–	+
Gluconate	+	+	–	+	ND
Lactate	–	–	–	–	+
L-Histidine	–	–	–	+	ND
Malate	+	–	+	+	+
D-Mannitol	+	–	+	–	ND
D-Sorbitol	+	–	+	+	ND
N-Acetyl-D-glucosamine	+	+	–	+	+
Salicin	+	–	–	+	ND
Peptidoglycan type	L-Orn–Gly ₂	L-Orn–Gly ₂	L-Orn–Gly ₂ *	L-Orn	L-Orn–Gly _{2–3}
Predominant fatty acids	C _{16:1} ω7c, C _{16:0}	C _{16:1} ω7c, C _{16:0}	C _{15:1} , C _{16:1} ω7c, C _{17:1}	C _{16:1} ω7c, C _{15:1} , C _{16:0}	C _{16:1} ω7c, C _{16:0} , C _{17:1} ω8c
DNA G + C content (mol%)	68.3	68.4	68.4–69.4†	65.8	67.0

*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-08^T, PB314^T and *Deinococcus radiodurans* R1. Isolate Ho-08^T was approximately ten times more sensitive than strain PB314^T 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-08^T and PB314^T, 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-08^T and PB314^T 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 Stanek & 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 PB314^T and Ho-08^T contained L-ornithine, alanine, glycine and glutamic acid. From the two-dimensional TLC patterns of peptides in partial peptidoglycan hydrolysates, strains PB314^T and Ho-08^T contained peptidoglycan type A3β (L-Orn-Gly-Gly). MK-8 was the predominant respiratory quinone of both strains PB314^T and Ho-08^T, 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 PB314^T and Ho-08^T were hexadecenoic acid (C_{16:1}ω7c; 49.9 and 51.8 %, respectively) and hexadecanoic acid (C_{16:0}; 32.3 and 37.3 %), 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 PB314^T and Ho-08^T and their phylogenetically closest relatives. In particular, in contrast to *D. grandis* DSM 3963^T, *D. indicus* MTCC4913^T and *D. radiodurans* ATCC 13939^T, they produced C_{18:0} but not C_{15:1}ω6c or C_{17:1}ω6c and contained smaller amounts of C_{17:0}. The fatty acid profile might be also useful for differentiation of the isolates. Strain Ho-08^T differed from PB314^T by the absence of C_{15:0}, C_{15:0} iso, C_{17:0} iso, C_{15:1} and C_{16:1}ω9c. The G + C contents of the genomic DNA of strains PB314^T and Ho-08^T 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 PB314^T and Ho-08^T 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-08^T. 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 PB314^T and Ho-08^T showed unknown phosphoglycolipid PGL₁, glycolipids GL₁

Fig. 2. Representative survival curves for strain PB314^T (▲) and strain Ho-08^T (■) following exposure to gamma radiation. Survival of *D. radiodurans* R1 (●) is also shown. Each increment on the y-axis represents a tenfold reduction in viability. Error bars represent the range of values of duplicate experiments.

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 PB314^T and Ho-08^T 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 PB314^T to strain Ho-08^T was 22 %, which is low enough to differentiate strain PB314^T and strain Ho-08^T as members of different species (Wayne *et al.*, 1987).

16S rRNA gene sequence comparison showed that strains PB314^T and Ho-08^T 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 3963^T, *D. indicus* Wt/1a^T and *D. radiodurans* DSM 20539^T. The presence of peptidoglycan type A3 β 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 PB314^T and Ho-08^T showed all the typical characters 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 PB314^T and Ho-08^T 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 (a.qua'ti.cus. L. masc. adj. *aquaticus* living, growing or found in or by water, aquatic).

Cells are Gram-negative, non-spore-forming rods, 2.5–3.0 μ m 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-hydroxybuty-

rate, 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 C_{16:1} and C_{16:0}. 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, PB314^T (=KCTC 12552^T =NBRC 101311^T), 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 μ m 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 C_{16:1} and C_{16:0}. 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-08^T (=KCTC 12553^T =NBRC 101312^T), 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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