Deinococcus piscis sp. nov., a radiation-resistant bacterium isolated from a marine fish

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A radiation-resistant, Gram-stain-positive, non-motile, non-sporulating, aerobic, coccoid bacterium, strain 3ax\textsuperscript{T}, was isolated from a marine fish (black pomfret, Parastromateus niger), with radiation as a selective pressure. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 3ax\textsuperscript{T} exhibited highest similarity (97.9\%) with Deinococcus proteolyticus DSM 20540\textsuperscript{T}. The $\Delta T_m$ for DNA–DNA hybridization of $D. \ proteolyticus$ DSM 20540\textsuperscript{T} and strain 3ax\textsuperscript{T} was 15.3 °C, indicating that the novel strain was distinct from $D. \ proteolyticus$ DSM 20540\textsuperscript{T}. The predominant respiratory quinone was MK-8. Strain 3ax\textsuperscript{T} could grow at 20–42 °C; the optimum temperature for growth was 35 °C. The strain grew well at pH 6–9, with optimum growth at pH 7. The cell wall contained ornithine. The major fatty acids were 16 : 0, 16 : 1\textsuperscript{c}, 18 : 1\textsuperscript{c}, and 18 : 1\textsuperscript{w}9c. Three phosphoglycerolipids and one aminophospholipid were the major polar lipids. Based on the genotypic, phenotypic and chemotaxonomic characteristics, strain 3ax\textsuperscript{T} was significantly different from $D. \ proteolyticus$ DSM 20540\textsuperscript{T} and, therefore, it was identified as representing a novel species of the genus Deinococcus, for which the name Deinococcus piscis sp. nov. is proposed. The type strain is 3ax\textsuperscript{T} (=MTCC9123\textsuperscript{T}=DSM 19767\textsuperscript{T}).

The species of the genus Deinococcus are known for their extreme radiation resistance. Since the first species, Deinococcus radiodurans, was reported, a further 32 species of this genus have been reported, isolated from diverse environmental sources such as continental Antarctica, the plant rhizosphere, deserts, hot springs, radioactive sites, industrial waste, animal faeces, laboratory contamination, animal tissues, water and environmental samples (Aker et al., 2008; de Groot et al., 2005; Ferreira et al., 1997; Im et al., 2008; Kämpfer et al., 2008; Lai et al., 2006; Rainey et al., 2005, 2007; Shashidhar & Bandekar, 2006; Suresh et al., 2004; Weon et al., 2007; Zhang et al., 2007). Recently, four psychrophilic Deinococcus species have been described from the treeline of alpine environments (Callegan et al., 2008). Two species, Deinococcus radiopugnans and Deinococcus radiophilus, were first isolated from marine fishes, haddock and Bombay duck, respectively (Murray, 1992). While carrying out a project to isolate novel radiation-resistant bacteria from the environment for their possible use in bioremediation, a novel Deinococcus strain (3ax\textsuperscript{T}) was isolated from a marine fish, black pomfret (Parastromateus niger).

Strain 3ax\textsuperscript{T} was isolated by using radiation stress. Various fish samples (25 g each) were exposed to radiation doses of 3–10 kGy from a 60Co source (Gamma Chamber 5000; BRIT) at a dose rate of 7 kGy h$^{-1}$. Irradiated samples (25 g) were homogenized in 225 ml TGY broth (1 % tryptone, 0.1 % glucose, 0.5 % yeast extract, w/v), 0.1 ml homogenate was spread on TGY agar and plates were incubated at 35 °C for 48 h. All the isolates obtained were streaked and purified. Each pure culture was grown to early stationary phase (18 h) in TGY broth and exposed to 5, 10 and 15 kGy. Cultures that survived doses above 5 kGy were characterized further. These studies led to the isolation of strain 3ax\textsuperscript{T}, which was highly resistant to ionizing radiation (>10 kGy); detailed characterization of this bacterium was carried out. The following description is based upon study of a single isolate and no other phylogenetically closely related strain was isolated.

DNA isolation was carried out as described by Earl et al. (2002). The 16S rRNA gene was amplified, purified and sequenced as described by Shashidhar & Bandekar (2006). 16S rRNA gene sequence identity was determined using BLAST (BLASTN and MEGABLAST) at the National Center for Biotechnology Information website (http://www.ncbi.nih.gov/blast/). The partial 16S rRNA gene sequence containing 1464 bp was aligned against representative reference sequences of members of the genus Deinococcus and related taxa by using MEGA version 4 (Tamura et al., 2007). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Stability among clades of the
A phylogenetic tree was assessed by taking 1000 bootstrap replicates of the dataset. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain 3axT was closely related to *Deinococcus proteolyticus* DSM 20540T, with a similarity of 97.9%. The topology of the phylogenetic tree showed that strain 3axT formed a clade with *D. proteolyticus* DSM 20540T, with a bootstrap resampling value of 100% (Fig. 1). When a phylogenetic tree was reconstructed with the UPGMA method using MEGA version 4 (Tamura *et al.*, 2007), strain 3axT again formed the closest relationship with *D. proteolyticus* DSM 20540T, with a bootstrap resampling value of 98% (Supplementary Fig. S1). Strain 3axT showed 16S rRNA gene sequence similarities in the range 89.1–97.9% to the type strains of recognized *Deinococcus* species. The 16S rRNA gene sequence of strain 3axT had the signature nucleotides reported for the genus *Deinococcus* (Rainey *et al.*, 1997). However, in strain 3axT, signature nucleotides C and C at positions 584 and 1471 were replaced by G and T, respectively. As shown in Fig. 1, *D. proteolyticus* DSM 20540T was found to be the closest relative of strain 3axT and therefore all comparative studies were carried out using *D. proteolyticus* DSM 20540T.

The fluorimetric thermal denaturation method was used for the estimation of DNA–DNA relatedness between strain 3axT and *D. proteolyticus* DSM 20540T. In brief, equal amounts of purified DNA of strain 3axT and *D. proteolyticus* DSM 20540T were mixed and denatured at 99 °C for 10 min in 0.1× SSC, followed by annealing at 80.15 °C for 8 h. After annealing, the temperature was brought down progressively to 25 °C (10 °C per hour) in a thermal cycler. Prior to performing thermal denaturation experiments of the hybrids and homologous DNA, SYBR Green I solution (1 : 100 000 final concentration) was added to the PCR tubes. The experiment was started in a real-time PCR machine (Corbett Rotor Gene 300) with a period of 15 min at room temperature (25 °C), followed by a ramp from 25 to 100 °C at 0.2 °C s⁻¹. Fluorescence measurements were performed at each step during this ramp (Gonzalez & Saiz-Jimenez, 2005). Tm values of total genomic DNA from homologous and hybrid solutions were calculated as the temperatures corresponding to 50% decrease in fluorescence. The graph was plotted by taking the mean values of three independent experiments.

DNA hybridization is regarded as the reference method to distinguish between bacterial species. Wayne *et al.* (1987) defined bacterial species as a group of strains (including the type strain) that share 70% or greater DNA–DNA relatedness with ΔTm of 5 °C or lower. The ΔTm between the homologous DNA solution of *D. proteolyticus* DSM 20540T and hybrid DNA solution of strain 3axT and *D. proteolyticus* DSM 20540T was 15.3 ± 2.2 °C (Supplementary Fig. S2), corresponding to approx. 15% DNA–DNA relatedness (Gonzalez & Saiz-Jimenez, 2005). This value is much greater than the cut-off of 5 °C suggested by Wayne *et al.* (1987) for the definition of bacterial species. Therefore, strain 3axT is distinct from *D. proteolyticus* DSM 20540T.

**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain 3axT and recognized *Deinococcus* species. The dendrogram was constructed from distance matrices according to the neighbour-joining method. Numbers at branching points represent bootstrap percentages based on 1000 replicates. The sequence of *Truepera radiovictrix* RQ-24T was used as the outgroup. Bar, 2 inferred substitutions per 100 nucleotide positions.
Radiation-resistance and taxonomic comparisons were made between strain 3axT and *D. proteolyticus* DSM 20540T. For morphological, physiological and biochemical studies, the two strains were grown to exponential phase (18 h) in TGY broth on a shaker incubator (100 r.p.m.) at 35 °C. Morphology was studied using phase-contrast microscopy during different growth phases. Cell size was measured using a micrometer (Crucikshank, 1972). Motility was studied by the hanging drop method. Biochemical tests were performed as described by Murray (1992) and Crucikshank (1972). The ranges of temperature (16–45 °C at intervals of 4 °C) and pH (5–11 at intervals of 1 pH unit) for growth and growth in the presence of salt (1, 3, 3.5, 4 and 5 % NaCl, w/v) were determined in TGY broth. Acid production from sugars was examined as described by Crucikshank (1972) with a few modifications: peptone water was supplemented with 0.01 % yeast extract, and results were recorded after 48 h. The biochemical and physiological properties of strain 3axT are detailed in the species description and in Table 1.

Radiation resistance is one of the distinguishing characters of the genus *Deinococcus* (Murray, 1992). Radiation-survival experiments were carried out as described previously (Shashidhar & Bandekar, 2006). The decimal reduction dose (D10), the dose required to reduce the cell number by 90 %, was calculated from the survival curve. Strain 3axT showed high resistance to ionizing radiation, with a characteristic shoulder in the gamma-radiation survival curve. The D10 for *D. proteolyticus* DSM 20540T and strain 3axT were 10 and 7.4 kGy, respectively.

Fatty acid methyl ester analysis was carried out by the DSMZ Identification Service. Extraction, separation and identification of isoprenoid quinones was carried out as described by Reddy et al. (2003). Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987). Polar lipids were extracted and analysed by TLC according to the method of Tindall (1991).

The major fatty acids present in strain 3axT were 16:0, 16:1o7c, 16:1o9c and 18:1o9c (Supplementary Table S1). The fatty acid composition of strain 3axT was quantitatively and qualitatively different from that of *D. proteolyticus* DSM 20540T, iso-17:1o9c and 16:1o5c, present in *D. proteolyticus* DSM 20540T, were absent from strain 3axT (Supplementary Table S1). These differences distinguish strain 3axT from its phylogenetically closest relative *D. proteolyticus* DSM 20540T. The peptidoglycan of strain 3axT contained ornithine, which is an important chemotaxonomic marker for the genus *Deinococcus* (Murray, 1992). The main respiratory quinone was MK-8. The polar lipids of 3axT were dominated by phosphoglycolipids (PGL). The major polar lipids PGL1, PGL2 and PGL3 of 3axT were similar to those of *D. proteolyticus* DSM 20540T (Supplementary Fig. S3). Also, three phospholipids (PL2, PL3 and PL4) and one unidentified polar lipid (L3) present in 3axT showed the same mobility as components found in *D. proteolyticus* DSM 20540T. One aminophospholipid, a glycolipid (GL2) and a phospholipid (PL1) were unique to strain 3axT. Two unidentified polar lipids (L1 and L2), one glycolipid (GL1), one aminoglycolipid (AGL) and two phospholipids (PL1 and PL5) present in *D. proteolyticus* DSM 20540T were absent from strain 3axT. These results indicate that the polar lipid profile of 3axT is different from that of *D. proteolyticus* DSM 20540T. The major polar lipids in strain 3axT were glycolipids and phosphoglycolipids, as observed in other *Deinococcus* species (Counsell & Murray, 1986) (Supplementary Fig. S3).

Strain 3axT showed distinct morphological, physiological and chemotaxonomic characteristics typical of the genus *Deinococcus*. These results substantiate the inclusion of strain 3axT within the genus *Deinococcus*. Even though the 16S rRNA gene sequence of strain 3axT showed 97.9 % similarity to that of *D. proteolyticus* DSM 20540T, it differed significantly from *D. proteolyticus* DSM 20540T with respect to DNA–DNA hybridization (ΔTm > 5 °C). The unique phylogenetic position, distinct fatty acid and polar lipid compositions and phenotypic characters (Supplementary Table S2) suggest that strain 3axT represents a novel species of the genus *Deinococcus*, for which the name *Deinococcus piscis* sp. nov. is proposed.

**Description of *Deinococcus piscis* sp. nov.**

*Deinococcus piscis* (pis’cis. L. gen. masc. n. piscis of a fish).

Aerobic, Gram-stain-positive, non-spore-forming, non-motile diplococci. Cells are 1–1.5 μm in diameter. Colonies on TGY agar medium are pale-pink coloured, smooth, convex and circular with uniform edges, 1–2 mm in diameter. Optimum growth at 35 °C and pH 7. Grows in the presence of 4 % NaCl. Highly resistant to gamma radiation (D10 7.4 kGy). The respiratory quinone is MK-8. The major fatty acids are 16:0, 16:1o7c, 16:1o9c and

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The cell-wall peptidoglycan contains ornithine. Positive for catalase and oxidase. Hydrolyses gelatin and casein; negative for amylase, urease, tryptophan deaminase and β-galactosidase activities. Does not produce indole or reduce nitrate. Sensitive to H₂O₂ (2 mM). Does not hydrolyse aesculin. Produces acid from maltose under both aerobic and anaerobic conditions; acid production under aerobic conditions was observed from sucrose, fructose and glucose in peptone water supplemented with 0.01 % yeast extract. Does not produce acid from adonitol, sorbitol, melibiose or mannose. Major polar lipids are three phosphoglycolipids, one aminophospholipid, four phospholipids, one unidentified lipid and one glycolipid.

The type strain, 3axT (=MTCC9123T =DSM 19767T), was isolated from a marine fish (black pomfret, Paenarasmus niger).

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References


