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 3axT, 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 3axT exhibited highest similarity (97.9 %) with Deinococcus proteolyticus DSM 20540T. The ΔTm for DNA–DNA hybridization of D. proteolyticus DSM 20540T and strain 3axT was 15.3 °C, indicating that the novel strain was distinct from D. proteolyticus DSM 20540T. The predominant respiratory quinone was MK-8. Strain 3axT 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 : 1w7c, 16 : 1w9c and 18 : 1ω9c. Three phosphoglycolipids and one aminophospholipid were the major polar lipids. Based on the genotypic, phenotypic and chemotaxonomic characteristics, strain 3axT was significantly different from D. proteolyticus DSM 20540T 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 3axT (=MTCC9123T=DSM 19767T).

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 radiougranus 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 (3axT) was isolated from a marine fish, black pomfret (Parastromateus niger).

Strain 3axT 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 3axT, 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.nlm.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
phylogenetic tree was assessed by taking 1000 bootstrap replicates of the dataset. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain 3ax\(^T\) was closely related to *Deinococcus proteolyticus* DSM 20540\(^T\), with a similarity of 97.9%. The topology of the phylogenetic tree showed that strain 3ax\(^T\) formed a clade with *D. proteolyticus* DSM 20540\(^T\), 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 3ax\(^T\) again formed the closest relationship with *D. proteolyticus* DSM 20540\(^T\), with a bootstrap resampling value of 98% (Supplementary Fig. S1). Strain 3ax\(^T\) 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 3ax\(^T\) had the signature nucleotides reported for the genus *Deinococcus* (Rainey et al., 1997). However, in strain 3ax\(^T\), 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 20540\(^T\) was found to be the closest relative of strain 3ax\(^T\) and therefore all comparative studies were carried out using *D. proteolyticus* DSM 20540\(^T\).

The fluorimetric thermal denaturation method was used for the estimation of DNA–DNA relatedness between strain 3ax\(^T\) and *D. proteolyticus* DSM 20540\(^T\). In brief, equal amounts of purified DNA of strain 3ax\(^T\) and *D. proteolyticus* DSM 20540\(^T\) 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 *DTm* of 5 °C or lower. The *DTm* between the homologous DNA solution of *D. proteolyticus* DSM 20540\(^T\) and hybrid DNA solution of strain 3ax\(^T\) and *D. proteolyticus* DSM 20540\(^T\) 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 3ax\(^T\) is distinct from *D. proteolyticus* DSM 20540\(^T\).
Radiation-resistance and taxonomic comparisons were made between strain 3ax<sup>T</sup> and <i>D. proteolyticus</i> DSM 20540<sup>T</sup>. 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 (Cruickshank, 1972). Motility was studied by the hanging drop method. Biochemical tests were performed as described by Murray (1992) and Cruickshank (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 Cruickshank (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 3ax<sup>T</sup> are detailed in the species description and in Table 1.

Radiation resistance is one of the distinguishing characters of the genus <i>Deinococcus</i> (Murray, 1992). Radiation-survival experiments were carried out as described previously (Shashidhar & Bandekar, 2006). The decimal reduction dose (D<sub>10</sub>), the dose required to reduce the cell number by 90 %, was calculated from the survival curve. Strain 3ax<sup>T</sup> showed very high resistance to ionizing radiation, with a characteristic shoulder in the gamma-radiation survival curve. The D<sub>10</sub> for <i>D. proteolyticus</i> DSM 20540<sup>T</sup> and strain 3ax<sup>T</sup> 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 3ax<sup>T</sup> were 16:0, 16:1<sup>ω7c</sup>, 16:1<sup>ω9c</sup> and 18:1<sup>ω9c</sup> (Supplementary Table S1). The fatty acid composition of strain 3ax<sup>T</sup> was quantitatively and qualitatively different from that of <i>D. proteolyticus</i> DSM 20540<sup>T</sup>, iso-17:1<sup>ω9c</sup> and 16:1<sup>ω5c</sup>, present in <i>D. proteolyticus</i> DSM 20540<sup>T</sup>, were absent from strain 3ax<sup>T</sup> (Supplementary Table S1). These differences distinguish strain 3ax<sup>T</sup> from its phylogenetically closest relative <i>D. proteolyticus</i> DSM 20540<sup>T</sup>. The peptidoglycan of strain 3ax<sup>T</sup> contained ornithine, which is an important chemotaxonomic marker for the genus <i>Deinococcus</i> (Murray, 1992). The main respiratory quinone was MK-8. The polar lipids of 3ax<sup>T</sup> were dominated by phosphoglycolipids (PGL). The major polar lipids PGL1, PGL2 and PGL3 of 3ax<sup>T</sup> were similar to those of <i>D. proteolyticus</i> DSM 20540<sup>T</sup> (Supplementary Fig. S3). Also, three phospholipids (PL2, PL3 and PL4) and one unidentified polar lipid (L3) present in 3ax<sup>T</sup> showed the same mobility as components found in <i>D. proteolyticus</i> DSM 20540<sup>T</sup>. One aminophospholipid, a glycolipid (GL2) and a phospholipid (PL1) were unique to strain 3ax<sup>T</sup>. Two unidentified polar lipids (L1 and L2), one glycolipid (GL1), one aminoglycolipid (AGL) and two phospholipids (PL1 and PL5) present in <i>D. proteolyticus</i> DSM 20540<sup>T</sup> were absent from strain 3ax<sup>T</sup>. These results indicate that the polar lipid profile of 3ax<sup>T</sup> is different from that of <i>D. proteolyticus</i> DSM 20540<sup>T</sup>. The major polar lipids in strain 3ax<sup>T</sup> were glycolipids and phosphoglycolipids, as observed in other <i>Deinococcus</i> species (Counsell & Murray, 1986) (Supplementary Fig. S3).

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

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

<i>Deinococcus piscis</i> (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 (D<sub>10</sub> 7.4 kGy). The respiratory quinone is MK-8. The major fatty acids are 16:0, 16:1<sup>ω7c</sup>, 16:1<sup>ω9c</sup> and

### Table 1. Differential characteristics of strain 3ax<sup>T</sup> and <i>D. proteolyticus</i> DSM 20540<sup>T</sup>

<table>
<thead>
<tr>
<th>Test</th>
<th>Strain 3ax&lt;sup&gt;T&lt;/sup&gt;</th>
<th>&lt;i&gt;D. proteolyticus&lt;/i&gt; DSM 20540&lt;sup&gt;T&lt;/sup&gt;</th>
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<tr>
<td>Asesculin hydrolysis</td>
<td>–</td>
<td>+</td>
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<td>Resistance to 2 mM H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth at 4 % NaCl</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Starch hydrolysis</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Tryptophan deaminase</td>
<td>–</td>
<td>+</td>
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<td>Acid production (aerobic/anaerobic) from:</td>
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<td>Adonitol</td>
<td>–/–</td>
<td>+/+</td>
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<td>Sorbitol</td>
<td>–/–</td>
<td>–/+</td>
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<td>Maltose</td>
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<td>Melibiose</td>
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<td>Glucose</td>
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<td>Sucrose</td>
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<td>Fructose</td>
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18:1ω9c. The cell-wall peptidoglycan contains ornithine. Positive for catalase and oxidase. Hydrolyses gelatin and casein; negative for amylase, urease, tryptophan deaminase and ß-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 phosphoglycerolipids, one aminophospholipid, four phospholipids, one unidentified lipid and one glycolipid.

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

Acknowledgements

We are grateful to Professor Dr Erko Stackebrandt, DSMZ, for providing D. proteolyticus DSM 20540T. We thank Dr J. P. Ezubey, Laboratoire de Bactériologie, École Nationale Vétérinaire Toulouse, for providing the etymology of the species epithet.

References


