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Deinococcus rubrus sp. nov., a Bacterium Isolated from Antarctic Coastal Sea Water

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Two Gram-staining-negative, red-pinkish, coccus-shaped, non-motile, and aerobic bacterial strains, designated Ant21^T and Ant22, were isolated from the Antarctic coastal sea water. Strains Ant21^T and Ant22 showed UVC and gamma radiation resistance. Phylogenetic analyses based on 16S rRNA gene sequences determined that these strains belong to the genus *Deinococcus*. Through the analyses of the 16S rRNA gene sequences, strains Ant21^T and Ant22 were found to have 97.7% and 97.8% similarity to *Deinococcus marmoris* DSM 12784^T and 97.0% and 97.2% similarity to *Deinococcus saxicola* AA-1444^T, respectively. The sequence similarity with the type strains of other *Deinococcus* species was less than 96.9% for both strains. Strains Ant21^T and Ant22 shared relatively high 16S rRNA gene sequence similarity (99.3%) and had a closely related DNA reassociation value of 84 ± 0.5%. Meanwhile, they showed a low level of DNA-DNA hybridization (<30%) with other closely related species of the genus *Deinococcus*. The two strains also showed typical chemotaxonomic features for the genus *Deinococcus*, in terms of the major polar lipid (phosphoglycolipid) and the major fatty acids ($C_{16:0}$, $C_{16:1}$ *ω6c*/*ω7c*, iso-$C_{17:0}$, and iso-$C_{15:0}$). They grew at temperatures between 4°C and 30°C and at pH values of 6.0–8.0. Based on the physiological characteristics, the 16S rRNA gene sequence analysis results, and the low DNA-DNA reassociation level with *Deionococcus marmoris*, strains Ant21^T (= KEMB 9004-167^T =JCM 31436^T) and Ant22 (KEMB 9004-168 =JCM 31437) represent novel species belonging to the genus *Deinococcus*, for which the name *Deinococcus rubrus* is proposed.

Keywords: *Deinococcus*, radiation resistance, taxonomy, coastal sea water

Introduction

Species in the genus *Deinococcus* are characterized by their resistance to UVC, gamma radiation, and desiccation [1–7]. Ionizing radiation creates reactive oxygen species, which can damage DNA, in both prokaryotic and eukaryotic cells. Radiation-resistant microorganisms contain special enzymes that can protect and repair the damaged DNA after irradiation [2, 8–11]. During research on the bacterial community of the Antarctic coastal sea water, two strains of *Deinococcus*-like bacteria were isolated. At present, the genus *Deinococcus* comprises more than 58 species (http://www.bacterio.net). The taxonomy of the genus has been described extensively [12, 13], and research related to the genus *Deinococcus* is still growing. Several novel *Deinococcus* strains have been isolated from the soil, food, feces, and dust.

In this study, we report the taxonomic characteristics of two novel bacterial strains, Ant21^T and Ant22, isolated from Antarctic coastal sea water (S72°58′ 16.3″, W60°22′ 51.1″). The characteristics of strains Ant21^T and Ant22 were studied, and both strains were identified as a novel species

of the genus *Deinococcus*.

Materials and Methods

Isolation

Strains Ant21^T and Ant22 were isolated from the costal sea water collected at the Antarctic region during 2014. The sea water sample was irradiated with 3 kGy using a cobal-60 gamma irradiator (point source-AECL, IR-79), and 100 µl aliquots spread onto 10-times diluted R2A agar (Difco, USA) and incubated at 20°C for 1 week. The colonies were purified by transferring them onto new plates and incubating them once again under the same conditions. The isolates were routinely cultured on R2A agar (Difco) at 20°C and maintained as a glycerol suspension (20% (w/v)) at −70°C.

For comparative taxonomic research, closely related type strains (*Deinococcus marmoris* KACC 12218^T and *Deinococcus saxicola* KACC 12240^T) were obtained from the Korean Agricultural Culture Collection (Korea) and grown under identical conditions throughout the experiment.

16S rRNA Gene Sequencing and Phylogenetic Analyses

The genomic DNA was isolated using CTAB/NaCl solution [14]. The 16S rRNA gene was amplified using universal bacterial primers 27F and 1492R under the following conditions: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min, with a final extension step at 72°C for 5 min [15]. The 16S rRNA genes were sequenced at Macrogen Inc. (Korea) using an Applied Biosystems model 3730XL automated DNA sequencing system, and using 27F, 785F, 907R, and 1492R universal bacterial primers (Applied Biosystems, USA). The 16S rRNA gene sequences for strains Ant21^T and Ant22 were compared against those of prokaryotic type strains using the NCBI BLAST (www.ncbi.nlm.nih.gov/blast/). The 16S rRNA gene sequences of the related taxa were obtained from GenBank and edited using the BioEdit program [16]. The phylogenetic tree was drawn by using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods [17], and evolutionary distances were calculated using the Kimura two-parameter model [18] in the MEGA5 program [19] with bootstrap values based on 1,000 replications [20].

Morphological, Physiological, and Biochemical Characterization

Growth at different temperatures (4°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 37°C) was tested using R2A agar (Difco) for 5 days. Growth at various pH levels (4, 5, 6, 7, 8, 9, and 10) was determined using R2A broth (MBcell, Korea) at 20°C. To maintain the pH of the medium, three buffers were used (final concentration of 50 mM): acetate buffer (for pH 4.0–5.0), phosphate buffer (for pH 6.0–8.0), and Tris buffer (for pH 9.0–10.0). NaCl tolerance was assessed in R2A broth (MBcell) supplemented with 0, 0.5, 1–10% (w/v) NaCl (1% intervals) at 20°C. Cellular morphology and motility were observed by transmission electron microscopy and light microscopy (Neoscience NB-2000B, Korea). The colonial morphology, size, and color were observed with cells grown aerobically on R2A medium at 20°C for 5 days. The Gram-staining reaction was performed according to the classic Gram procedure described by Doetsch [21]. Oxidase activity was investigated through the oxidation of 1% (w/v) *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine and catalase activity was confirmed by measuring bubble production after applying 3% (v/v) hydrogen peroxide solution. Other enzymatic activities and carbon source utilization were examined by using the API 20NE, API 50CH, and API ZYM microtest systems and results were analyzed according to the recommendations of the manufacturers (bioMérieux, France). Growth on different media was also tested on trypticase soy agar (TSA; Difco), nutrient agar (Difco), and Luria-Bertani agar (Difco). A cobalt-60 based gamma irradiator was used to irradiate the strains with gamma rays [10]. After irradiation, the strains were inoculated onto R2A plates. For ultraviolet radiation, the cells were diluted and inoculated on five different R2A plates depending on UVC radiation dose. A UVC ultraviolet Crosslinker (UVP, CX-r2000, USA) at 254 nm was used, and different doses were applied [6] to each plate. A positive control, *Deinococcus radiodurans* R1^T (=DSM 20539^T), and a negative control, *Escherichia coli* K12 (=KCTC 1116), were both used to compare the survival rate [22, 23]. The survival rate after exposure to gamma and UVC radiation was analyzed with the early stationary phase (~10^7 CFU/ml) cells in TGY broth (Difco) [24]. The colony-forming units (CFU) of the strains were numbered, and the survival rate was determined.

Chemotaxonomy

Extracted polar lipids were examined by two-dimensional TLC on silica gel 60 F254 plates (20 × 20 cm, Merck, Germany). Chloroform/methanol/water (65:25:4, by vol.) was used as the first direction of the mobile phase, and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) was used as the second direction of the mobile phase. The different spots were determined by using specific reagents: 10% ethanolic molybdo-phosphoric acid (for total lipids), phosphomolybdic acid (for phospholipids), ninhydrin reagent (for aminolipids), and naphthol/sulfuric acid (for glycolipids) [25]. The fatty acid profiles were examined using cells grown on TSA for 72 h at 20°C. Two full loops of the early stationary growth phase cells were harvested, saponified, methylated, and extracted [26]. The fatty acids were analyzed using the microbial identification software packaged with the Hewlett Packard 6890 capillary GLC, the Sherlock system MIDI 6.0, and the Sherlock aerobic bacterial database (TSBA6) [27].

Genomic Analysis

The genomic DNA was extracted as described above and enzymatically degraded into nucleosides. The nucleosides were analyzed using HPLC and the DNA G+C contents were calculated [28]. The DNA-DNA reassociation experiment was performed

fluorometrically using photobiotin-labeled DNA probes and micro-dilution wells [29]. Hybridization was examined in the presence of 50 % (v/v) formamide with the ionic strength of 0.3 M NaCl and was performed reciprocally with five replications per sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized for the calculation of reassociation average values.

Results and Discussion

Cells of strains Ant21^T and Ant22 were Gram-staining-negative, non-motile, non-spore-forming, and coccus shaped, with a diameter of 1.5 μm (Fig. S1). Colonies on R2A were circular, smooth, and reddish pink in color. Growth occurred at 4–30°C and the optimum temperature for growth was 20ºC. The pH range for growth was pH 6.0–8.0, with an optimum of pH 7.0. Cells tolerated up to 4% (w/v) NaCl. Growth occurred on TSA, Luria-Bertani agar, and nutrient agar. The strains were oxidase negative and catalase positive. Strains Ant21^T and Ant22 showed gamma radiation resistance and UVC radiation resistance (Figs. S2 and S3), which are the characteristic features of the genus *Deinococcus* [2, 5, 22, 23]. The morphological, cultural, physiological, and biochemical characteristics are shown in the species description and Table 1.

The 16S rRNA gene sequence analysis results showed that strains Ant21^T and Ant22 belonged to the genus *Deinococcus*. strain Ant21^T showed the closest relationship to *Deinococcus marmoris* DSM 12784^T (97.7%), followed by *Deinococcus saxicola* AA-1444^T (97.0%) and *Deinococcus frigens* AA-692^T (96.9%). Sequence similarity to other *Deinococcus* species was <96.9%. In the neighbor-joining phylogenetic tree (Fig. 1), strains Ant21^T and Ant22 formed a monophyletic group with *Deinococcus marmoris* and combined the cluster of the genus *Deinococcus* with a high bootstrap value of 86%. This was also confirmed in the maximum-parsimony and maximum-likelihood algorithms (Figs. S4 and S5).

The major fatty acids of strains Ant21^T and Ant22 were 16:0, 16:1 *ω6c*/*ω7c*, 17:0 iso, and 15:0 iso, which are common fatty acids of the genus *Deinococcus* (Table 2). Polar lipids of strain Ant21^T consisted of unknown phosphoglycolipids (PGL1–2) and glycolipids (GL1–4), a typical pattern for the genus *Deinococcus* [30]. The strain also contained an unknown aminophospholipid (Fig. S6).

The DNA G+C contents of the strains Ant21^T and Ant22 were 62.2% and 61.9%, respectively. A DNA-DNA reassociation experiment was performed with strains Ant21^T, Ant22, *Deinococcus marmoris* DSM 12784^T, and *Deinococcus saxicola* AA-1444^T. Strains Ant21^T and Ant22 exhibited low DNA relatedness with *Deinococcus marmoris* DSM 12784^T, with a similarity of 23 ± 0.5% and 26 ± 0.8%, respectively. They also had low similarity of 22 ± 0.9% and

Table 1. Major differential phenotypic characteristics of strains Ant21^T, Ant22, and the closest species *D. marmoris* DSM 12784^T, followed by *D. saxicola* KACC 12240^T.

Characteristic	1	2	3	4
Colony color	red-pink	red-pink	pink	pink
Nitrate reduction to NO_2	+	+	-	-
Nitrate reduction to N_2	-	-	+	-
Growth range				
Temperature (ºC)	4–30	4–30	4–25	4–37
NaCl (%)	0.5–1	0.5–1	0–1	0–1
pH	6–8	6–8	7–8	4–9
Enzyme activity				
β-Glucosidase	+	+	-	+
β-Glucosidase (aesculin hydrolysis)	-	+	-	-
Protease (gelatin hydrolysis)	-	-	+	-
Assimilation				
D-Cellobiose	+	+	-	-
D-Fructose	+	+	-	-
D-Galactose	+	+	-	-
D-Glucose	-	-	+	+
D-Maltose	+	+	-	-
D-Mannose	-	-	+	-
α-Methyl-D-glucoside	+	+	-	+
α-Methyl-D-mannoside	+	+	-	+
L-Rhamnose	-	-	+	-
Salicin	+	+	-	+
L-Sorbose	+	+	-	-
D-Trehalose	-	-	+	-
5-Ketogluconate	+	+	-	-
Mannitol	-	-	+	+
Sorbitol	-	-	+	-
Xylitol	+	+	-	-
DNA G+C content (mol%)	62.2	61.9	62.8	59.4

Strains: 1, Strain Ant21^T; 2, Strain Ant22; 3, *Deinococcus marmoris* DSM 12784^T; 4, *Deinococcus saxicola* KACC 12240^T. All data were generated under comparative conditions, and all data were from this study. All strains showed the following positive enzyme activities: acid phosphatase, alkaline phosphatase, α-chymotrypsin, cysteine arylamidase, esterase (C4, C8), β-galactosidase (ONPG, PNPG), α-glucosidase (starch hydrolysis), leucine arylamidase, napthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase. All strains showed the following negative enzyme activities: *N*-acetyl-β-glucosaminidase, α-fucosidase, β-glucuronidase, lipase (C14), and α-mannosidase; negative for indole production. +, positive; -, negative.

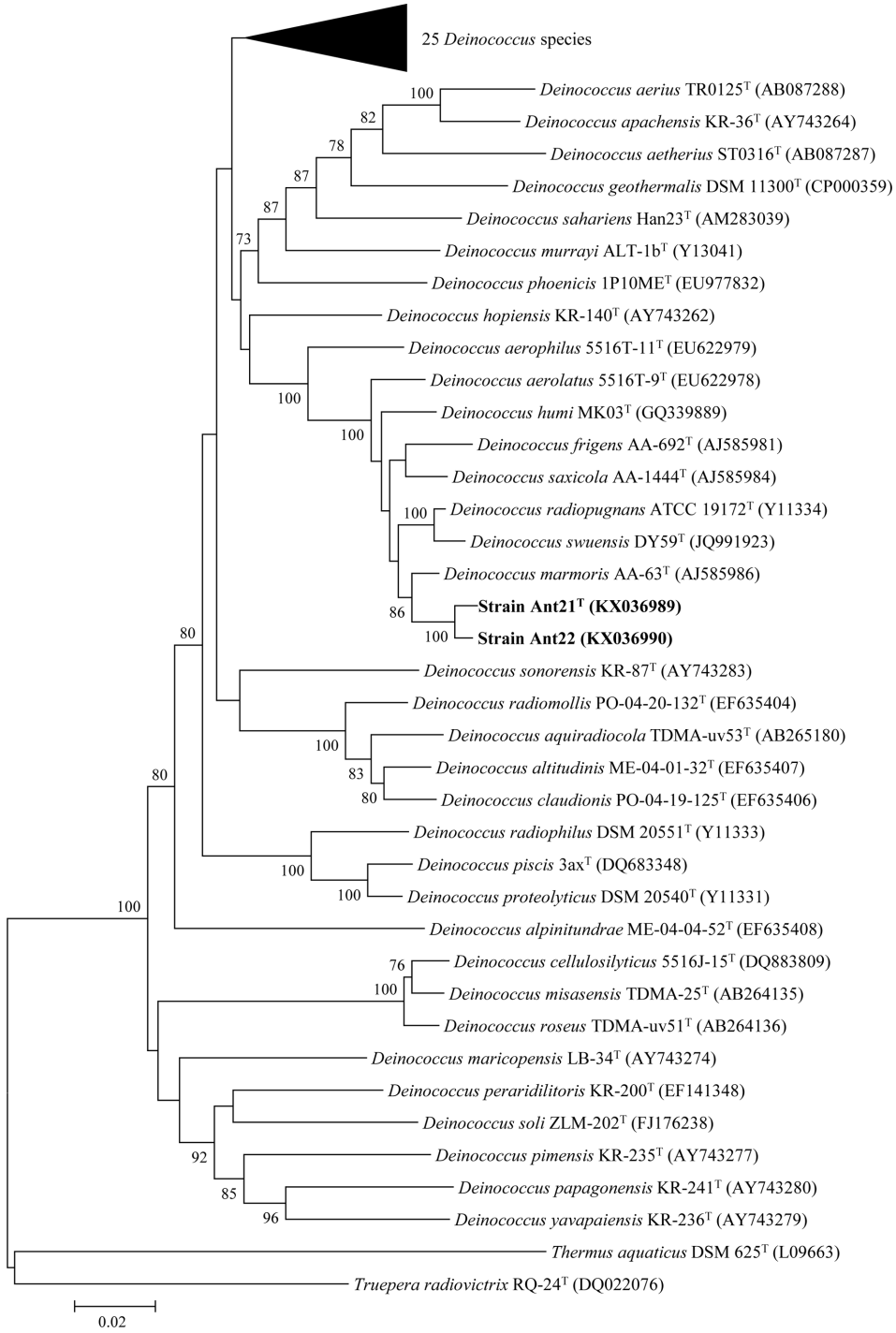

Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strains Ant21^T, Ant22, and other related species.

Truepera radiovictrix RQ-24^T (DQ022096) and *Thermus aquaticus* DSM 625^T (L09663) were used as the outgroup. Scale bar, 0.02 substitutions per position. Numbers at the nodes indicate the bootstrap values (greater than 70%) expressed as a percentage of 1,000 replicates.

25 ± 0.5% with *Deinococcus saxicola* AA-1444^T, respectively. Strain Ant21^T showed a high level of DNA reassociation with Ant22 (84 ± 0.5%). According to the phenotypic characters and DNA-DNA reassociation results (Table 3),

Table 2. Cellular fatty acid composition of strains Ant21^T, Ant22, and closely related strains.

Fatty acids	1	2	3	4
Saturated				
13:0 iso	3.4	3.0	2.1	tr
14:0	1.8	2.1	1.9	tr
15:0 iso	9.3	9.6	2.6	2.8
15:0 anteiso	1.2	1.4	2.8	4.0
16:0	10.6	12.1	7.3	5.0
17:0	2.7	2.2	4.0	6.0
17:0 iso	10.0	5.4	nd	1.9
17:0 iso 3OH	1.6	1.8	3.4	4.3
19:0	1.3	1.1	nd	tr
19:0 iso	1.6	1.8	3.7	3.5
19:0 cyclo *ω8c*	1.2	1.3	1.3	7.2
Unsaturated				
14:1 *ω5c*	4.0	4.8	7.9	nd
15:1 iso F	1.6	1.8	3.4	14.5
15:1 *ω6c*	7.6	6.2	4.7	21.7
15:1 *ω8c*	2.0	1.8	9.0	tr
16:1 *ω5c*	1.6	2.3	2.6	27.1
17: 1 *ω5c*	1.7	1.0	3.1	14.4
19:1 iso I	1.2	1.6	2.7	nd
Summed feature 1(13:0 3OH/15:1 i H)	2.0	2.1	nd	nd
Summed feature 3(16:1 *ω6c*/*ω7c*)	20.9	23.8	15.3	17.1

Strains: 1, Strain Ant21^T; 2, Strain Ant22; 3, *Deinococcus marmoris* AA-63^T; 4, *Deinococcus saxicola* AA-1444^T. All data are from this study. Fatty acids with >1% abundance are shown. nd, not detected; tr, trace < 1%.

strains Ant21^T and Ant22 seem to belong to the same species [29].

The chemotaxonomic characteristics of strains Ant21^T and Ant22 showed typical features of the genus *Deinococcus*, with the presence of the major fatty acids 16:0, 16:1 *ω6c*/*ω7c*, 17:0 iso, and 15:0 iso and major polar lipids that were unknown phosphoglycolipids. According to the analysis of the phylogenetic, chemotaxonomic, and phenotypic data, we demonstrated that strains Ant21^T and Ant22 represent a new species within the genus *Deinococcus*, for which the name *Deinococcus rubrus* sp. nov. is proposed.

Description of *Deinococcus rubrus* sp. nov.

Deinococcus rubrus (L. masc. adj. *rubrus*, red, referring to the color of the colony.)

Cells are Gram-staining-negative, catalase-positive, oxidase-negative, and aerobic. Cells are non-motile, coccus shaped, and 1.5 μm in diameter. Colonies are red-pink, circular, with entire margins after incubation on R2A at 20°C for 3 days. Cells grow at 4–30°C (optimum, 20°C), pH 6.0–8.0 (optimum, pH 7.0), and tolerate up to 4% (w/v) NaCl.

Table 3. DNA-DNA hybridization results between strains Ant21^T, Ant22, and closely related strains.

Strain	DNA hybridization (%) with			
	1	2	3	4
1. Ant21^T	100	84 ± 0.5	28 ± 0.8	21 ± 0.5
2. Ant22	87 ± 0.3	100	26 0.4	23 ± 0.3
3. *D.marmoris* AA-63^T	23 ± 0.5	26 ± 0.8	100	45 ± 0.3
4. *D.saxiocola* AA-1444^T	22 ± 0.9	24 ± 0.5	58 ± 0.3	100

Cells show positive enzyme activities for acid phosphatase, alkaline phosphatase, cystine arylamidase, α-chymotrypsin, esterase (C4), esterase (C8), β-galactosidase (ONPG), β-galactosidase (PNPG), α-glucosidase (starch hydrolysis), β-glucosidase, leucine arylamidase, naphtol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase. Cells show negative enzyme activities for *N*-acetyl-β-glucosamine, α-fucosidase, β-glucosidase (esculin hydrolysis), β-glucuronidase, lipase (C14), α-mannosidase, and protease (gelatin hydrolysis) (API ZYM and API 20NE).

Cells are positive for the assimilation of D-maltose and negative for the assimilation of *N*-acetyl-D-glucosamine, L-arabinose, D-glucose, D-mannitol, D-mannose, and urease (API 20NE). According to the API 50 CH tests, the cells are positive for acid production from D-arabitol, arbutin, D-cellobiose, esculin, D-fructose, D-galactose, 5-ketogluconate, D-maltose, α-methyl-D-glucoside, α-methyl-D-mannoside, salicin, L-sorbose, and xylitol. The cells are negative for acid production from *N*-acetylglucosamine, D-adonitol (ribitol), D-arabinose, L-arabinose, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, gluconate, D-glucose, glycerol, glycogen, inulin, 2-ketogluconate, D-lactose, D-lyxose, mannitol, D-mannose, melezitose, D-melibiose, β-methyl-D-xyloside, D-raffinose, L-rhamnose, D-ribose, sorbitol, D-tagatose, D-trehalose, turanose, and L-xylose.

The fatty acid profile included major amounts of 16:0, 16:1 *ω6c*/*ω7c*, 17:0 iso, and 15:0 iso. The polar lipids profile included major amounts of an unknown phosphoglycolipid. The G+C contents of strains Ant21^T and Ant22 are 62.2 mol% and 61.9 mol%, respectively. The isolated strains Ant21^T and Ant22 showed gamma and UVC radiation resistance with the D10 value of 3kGy and 400J/m^2, respectively.

The type strain Ant21^T (=KEMB 9004-167^T =JCM 31436^T) and strain Ant22 (=KEMB 9004-168 = JCM 31437) were isolated from Antarctic coastal sea water.

GenBank Accession Numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Deinococcus rubrus* Ant21^T and Ant22 are KX036989 and KX036990, respectively.

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