Isolation and Identification of Radiation-resistant Cocci Belonging to the Genus *Deinococcus* from Sewage Sludges and Animal Feeds

Hitoshi ITO, Hiroshi WATANABE, Masaaki TAKEHISA and Hiroshi IIZUKA*

Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma 370-12, Japan
**Institute of Applied Biology, Science University of Tokyo, Noda, Chiba 278, Japan*

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Six strains of radiation-resistant gram-positive cocci were isolated from sewage sludges and animal feeds in Japan after gamma-irradiation of more than 1.0 Mrad. All six strains were able to grow on nutrient agar slants, and some strains were also able to grow on glutamate agar slants. Cells of the six strains were single or diplococci, and occasionally seen in tetrads, being spheres averaging from 0.8 to 1.0 μm in diameter. The peptide subunit of cells of all the strains contained ornithine, and the predominant fatty acid component was a $C_{16:1}$. The GC content of the DNA of these strains ranged from 59 to 66 mol% thus indicating them as belonging to the genus *Deinococcus* Brooks and Murray 1981 which was previously called the "*Micrococcus radiodurans*" group. From the similar cultural characteristics and morphology, the six strains, TD1, TD3, TD9, T843, Fr 3 and Fr 7, were identified as *D. proteolyticus*. However, the predominant component of cellular fatty acids of strain T843 was similar to that of *D. radiodurans*.

The resistance to gamma-radiation of these new isolates was similar to that of *D. radiodurans* R_1, and D_{10} values in phosphate buffer ranged from 0.10 to 0.25 Mrad, and the low oxygen enhancement effect caused by radiation was distinct from other kinds of bacteria.

Recent taxonomic studies suggest that the radiation-resistant, red pigmented, catalase-positive cocci are distinct from conventional *Micrococcus* species.[1,2] Schleifer and Kandler reported[1] that the peptidoglycan in the cell wall contains a unique ornithine subunit, and the occurrence of a significant amount of a lipoprotein-polysaccharide complex on the outermost layer of "*Micrococcus radiodurans*" resembles that of gram-negative bacteria. Furthermore, the predominant fatty acid in lipid extracts of cells contained a $C_{16:1}$ component, and it was also reported by Girard[2] that this characteristic resembles that of gram-negative bacteria. From most of the detailed studies of described species of radiation-resistant cocci, the new generic name *Deinococcus* in the new family of *Deinococcaceae* was proposed by Brooks and Murray for these bacteria.[3] In a previous study, radiation-resistant cocci from sawdust culture media of mushrooms were isolated by Ito, and identified as "*Micrococcus radiodurans*."[4] Recently we also isolated a so-called radiation-resistant red *Micrococcus* from sewage sludges[5] and mixed animal feeds[6] after gamma-irradiation of more than 1 Mrad. All of these isolates were able to grow on nutrient agar, and sometimes they constituted the main residual flora of sewage sludges in winter and mixed animal feeds after high dose irradiation.

The present work describes isolation, identification and radiation-resistivities of typical isolates from sewage sludges and animal feeds after irradiation. All of the isolates belong to the genus *Deinococcus* and were identified as *Deinococcus proteolyticus*.

MATERIALS AND METHODS

1. Bacterial strains. Three strains, TD1, TD3 and TD9, were isolated from digested sewage sludge in the winter after gamma-irradiation at 2 Mrad. Strain T843 was isolated from activated sewage sludge in the spring after gamma-irradiation at 1.4 Mrad. Strains Fr3 and Fr7 were isolated from different kinds of mixed animal feeds after gamma-irradiation at 1 Mrad. Strains H48, H54 and H55 of "*M. radiodurans*" isolated from sawdust culture media were also used for comparison.[4] Strains of *Deinococcus radiodurans* R_1,[7] *Deinococcus radiophilus* CCM 2564,[8] *Deinococcus proteolyticus* CCM 2703[9] and *Micrococcus roseus* IFO 3764 were used for comparative purposes throughout the present investigation. These cultures were grown at 30°C on Difco-nutrient agar or PGYM agar containing 10 g of peptone, 2 g of glucose, 4 g of yeast extract, 10 g of meat extract, 2 g of sodium chloride and 20 g of agar per liter.

2. Determination of taxonomic characteristics. A taxonomic study of these isolates was carried out mainly by the method of Cowan and Steel[10] and identification was done in accordance with Bergey's Manual of Determinative Bacteriology, 8th Ed.,[11] and with the description of the taxa of *Deinococcus* provided by Brooks and Murray.[3] In addition, glutamate agar containing 5 g of sodium glutamate, 10 g of glucose, 1 g of K_2HPO_4, 0.1 g of KCl, 0.01 g of $FeSO_4$ and 20 g of agar per liter was used for the study of cultural characteristics. For the study of taxonomic characteristics of nutritionally fastidious strains, 0.1% yeast extract was added to each test medium.

Determination of the base composition of DNA by T_m and principal amino acids in the peptidoglycan were determined by the same method as described previously.[4]

The predominant cellular fatty acid component was determined by gas chromatography using a 3 m glass column of 15% diethyleneglycol succinate coated on 80/100 mesh Chromosorb GAW in an isothermal run at 200°C. The samples for gas chromatography were prepared by the method reported by Nishimura *et al.*[12]

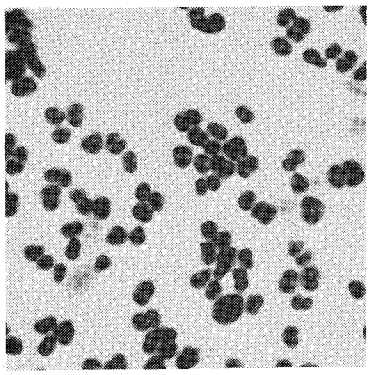
3. Radiation sensitivity. Pure cultures of each strain were grown for 40 hr in PGYM broth under constant aeration at 30°C. Cells at the stationary phase were harvested, washed twice with 0.067 M phosphate buffer, pH 7, and then resuspended in the same buffer. These suspensions concentrated to about 1×10^8 cells/ml were irradiated at *ca.* 25°C in equilibrium with atmospheric air (1.5 ml/1.5 cmϕ tube) or under bubbling with air through a capillary tube. Estimation of viable cell counts and dose rate measurements were carried out as described in previous papers.[4,13]

4. Measurement of catalase activity. Stationary phase cells of each strain incubated for 40 hr were collected and washed twice with distilled water, and lyophilized. Catalase activity was measured by the method reported in the previous report.[13]

RESULTS

1. Taxonomic characteristics

All of the isolates from sewage sludges and animal feeds were gram-positive, occurring as single or diplococci or tetrads. As shown in Fig. 1, cells of TD1 and Fr3 were diplococci, and occasionally formed tetrads morphologically resembling the R_1 strain of *D. radiodurans*, and strains TD3, TD9 and Fr7 tended to form diplococci similarly to *D. proteolyticus*. However, the cells of T843 occurred singly, and occasionally as diplococci. Cell sizes of all isolates were in the range of 0.8 to 1.0 μm which is smaller than that of *D. radiodurans* R_1. These six isolates were able to grow on nutrient agar slants, and this characteristic is distinct from *D. radiodurans* R_1 and *D. radiophilus* CCM 2564 as shown in Table I, and also distinct from H48, H54 and H55.[4] Strains TD1 and Fr7 were also able to grow on glutamate agar without a growth factor as well as *M. roseus*. All of these strains were strictly aerobic, oxidase-positive, catalase-positive and nonmotile, and some strains produced acids from sugars. The colonies of strains TD3, TD9 and Fr3 on nutrient agar plates after 3 days' incubation at 30°C were smooth, mucoid, raised and pale orange to orange in color. However, the color of T843 was white on nutrient agar, and that of TD1 and Fr3 was red as compared with the pink color of *D. proteolyticus* CCM 2703. On the other hand, the color of colonies of all isolates on PGYM agar plates was pale pink to orange red similar to that of *D. radiodurans* and other described species. A small amount of all strains from the agar slants gave a blue color reaction with concentrated sulphuric acid which should indicate the presence of carotenoid-like pigments in the cells.[4] Hydrolysis of gelatin and casein were observed in all of strains as well as other radiation-resistant cocci, and these characteristics are distinct from *M. roseus*. All six strains were able to grow on *ca.* 1% NaCl in media



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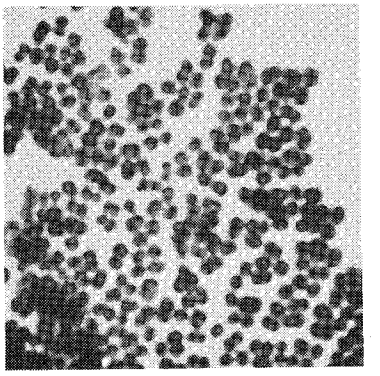

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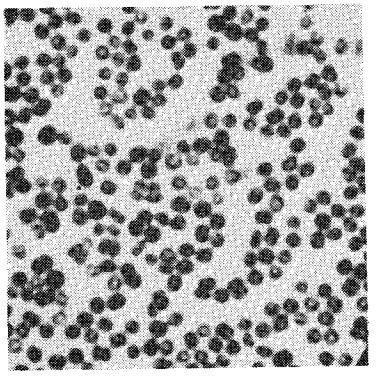

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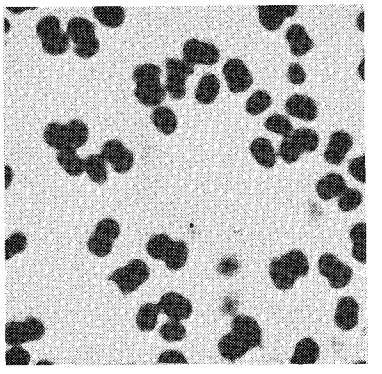

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FIG. 1. Phase Photomicrographs of Radiation-resistant Micrococci at the Exponential Phase of Growth on PGYM Agar. ×1500

1, strain TD1; 2, strain TD3; 3, strain T843; 4, *D. radiodurans* R_1.

and could not grow at 3% NaCl in media which is a similar characteristic to that of *D. radiodurans* R_1. Cells were resistant against lysis by lysozyme of all isolates, and they were lysed easily with the lytic enzyme of so-called "*Achromobacter lunatus*"[4,14] as well as other radiation-resistant cocci as shown in Table II.

Major amino acids found in the cell walls of the six isolates on paper chromatograms in a solvent system of phenol–water (7 : 3, v/v) are given in Fig. 2 with other described strains. The presence of ornithine, glycine, glutamic acid and alanine as main components of amino acids in peptidoglycan seems to justify identification of these six isolates as the same group of *D. radiodurans*. The predominant fatty acid compositions in lipid extracts of cells from these six isolates and three isolates from sawdust culture media indicated that these isolates can be divided into two clusters (Table III and Fig. 3). The predominant fatty acid of TD1, TD3, TD9, Fr3, Fr7, H48, *D. proteolyticus* CCM 2703 and *D. radiophilus* CCM 2564 was a $C_{16:1}$ component, and large amount of $C_{15:1}$ and $C_{16:1}$ were observed in strains of T843, H54, H55 and *D. radiodurans* R_1. The GC content of DNA of all these strains ranged from 59 to 66 mol% by T_m as shown in Table II, and these contents are lower than that of the strains of the genus *Micrococcus*.

Table I. Morphological and Cultural Characteristics of Radiation-resistant Cocci

	TD1	TD3	TD9	T843	Fr3	Fr7	*D. radiodurans* R_1	*D. radiophilus* CCM2564	*D. proteolyticus* CCM2703	*M. roseus* IFO 3764
Morphology of cells	Diplo or tetra	Diplo	Diplo	Single or diplo	Diplo or tetra	Diplo	Diplo or tetra	Diplo or tetra	Diplo	Single or diplo
Cell size (μm)	0.8~1.0	0.8	0.8~1.0	0.8~1.0	0.8~1.0	0.8	1.0~2.0	0.8	0.8~1.0	0.6~0.8
Color of colonies on nutrient agar	Red	Orange	Pale orange	White	Red	Pale orange	×	×	Pink	Pink
Growth on nutrient agar	+	+	+	+	+	+	−	−	+	+
Growth on glutamate agar	+	−	−	−	−	+	−	−	−	+
Growth at 1% NaCl	+	+	+	±	+	+	+	+	+	+
Growth at 3% NaCl	−	−	−	−	−	−	−	+	+	+

Diplo, diplococci; tetra, tetrads; ×, could not be observed.

TABLE II. BIOCHEMICAL CHARACTERISTICS OF RADIATION-RESISTANT COCCI

	TD1	TD3	TD9	T843	Fr3	Fr7	*D. radiodurans* R_1	*D. radiophilus* CCM2564	*D. proteolyticus* CCM2703	*M. roseus* IFO 3754
Gelatin liquefaction	++	+	+	+++	++	+	+	++	++	−
Hydrolysis of casein	+++	++	++	++	++	+++	++	++	++	−
NO_3 reduction	−	−	+++	−	−	+++	−	−	−	−
Hydrolysis of fats	+	−	−	+	+	−	−	−	−	−
Growth with novobiocin 0.6 μg/ml	−	−	−	−	−	−	−	−	−	−
Production of acid										
from glucose	−	−	+	+	−	−	+	+	+	+
xylose	−	−	−	−	−	−	−	−	−	−
sucrose	+	−	+	+	−	+	+	−	+	+
Lysis of cells by lysozyme	−	−	−	+	−	+	−	++	−	−
lytic enzyme of "*A. lunatus*"[14]	+	++	++	+++	+	++	++	+++	++	−
Oxidase	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Catalase	+++	+++	+++	+++	+++	+	+++	+++	+++	+++
GC content of DNA (%)	64	66	62	59	62	62	66	61	65	66～74[a]

+++, strong; ++, moderate; +, weak; −, negative.

[a] Values for *M. roseus*, with reference to Bergey's Manual of Determinative Bacteriology, 8th Ed.[11]

From these results, all radiation-resistant cocci are distinct from conventional *Micrococcus* species, and they should be included in the genus *Deinococcus* recently proposed by Brooks and Murray (1981). Here, all strains, TD1, TD3, TD9, T843, Fr3 and Fr7, should be identified as *D. proteolyticus*, which was formerly known as "*Micrococcus radio-proteolyticus*" isolated by Kobatake *et al.*,[9] on the basis of the similar cell morphology and ability to grow on nutrient agar. However, these six strains could not grow at 3% NaCl compared with strains CCM 2703, and TD9 and Fr7 had the ability to reduce nitrates to nitrites. In the case of strain T843, its predominant fatty acids were $C_{15:1}$ and $C_{16:1}$ similar to *D. radiodurans* R_1, whereas characteristics of occurring singly or in pairs, small cell size, white to pale pink colonies, 59% GC content of DNA and ability to grow on nutrient agar are distinct from those of *D. radiodurans*. On the other hand, isolates H54 and H55 from sawdust culture media should be classified as *D. radiodurans* from the similar

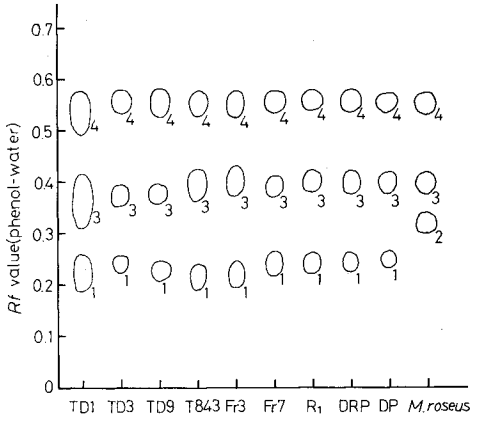

FIG. 2. Paper Chromatograms of Acid Hydrolysates (6 N HCl, 100°C, 8 hr) of Cell Walls of Radiation-resistant Micrococci Developed with Ninhydrin.

R_1, *D. radiodurans* R_1; DRP, *D. radiophilus* CCM 2564; DP, *D. proteolyticus* CCM 2703. 1, ornithine; 2, lysine; 3, glycine and glutamic acid; 4, alanine.

TABLE III. COMPOSITION OF PREDOMINANT CELLULAR FATTY ACIDS OF RADIATION-RESISTANT COCCI

Strain	Fatty acids (%)	
	$C_{15:1}$	$C_{16:1}$
TD1	10	80
TD3	10	83
TD9	12	82
T843	42	50
Fr3	10	80
Fr7	8	85
H48	12	78
H54	36	55
H55	40	52
D. radiodurans R_1	40	51
D. radiophilus CCM2564	6	80
D. proteolyticus CCM2703	5	80

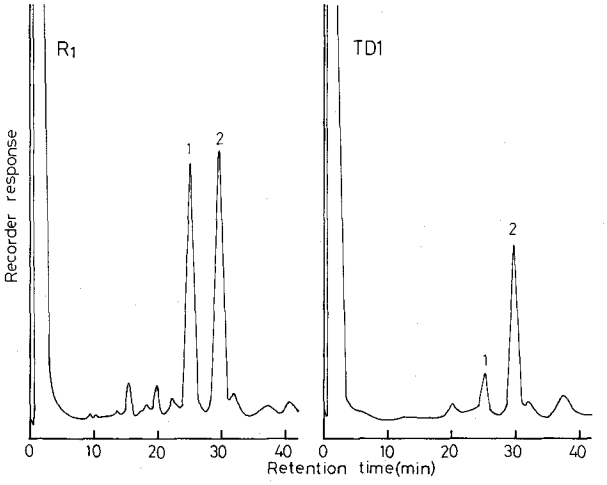

FIG. 3. Gas Chromatogram of Cellular Fatty Acids of *D. radiodurans* R_1 and Strain TD1.

1, $C_{15:1}$; 2, $C_{16:1}$.

TABLE IV. COMPARATIVE RADIATION SENSITIVITIES OF THE STRAINS OF *Deinococcus* AND THEIR CATALASE ACTIVITIES

Strain	D_{10} value (Mrad) with the condition of		Oxygen enhancement value	Catalase activity Kat. *f*
	Air-bubbling	Air-equilibrium		
TD1	0.20	0.20	1.0	99.9
TD3	0.10	0.15	1.5	90.7
TD9	0.18	0.20	1.1	84.4
T843	0.18	0.20	1.1	201.3
Fr3	0.13	0.17	1.3	78.6
Fr7	0.11	0.11	1.0	2.1
H48	0.19	0.22	1.2	23.7
H54	0.19	0.19	1.0	13.6
H55	0.22	0.22	1.0	38.0
D. radiodurans R_1	0.21	0.25	1.2	140.4
D. radiophilus CCM2564	0.22	0.24	1.1	81.0
D. proteolyticus CCM2703	0.34	0.34	1.0	217.2
M. roseus IFO 3764	0.03	0.06	2.0	95.5

predominant fatty acid components and other similar characteristics as described in the previous report.[4] However, strain H48 should be reidentified as *D. radiophilus* from the predominant fatty acid, $C_{16:1}$, small cell size, and similar growth characteristics such as the ability to grow at 3% NaCl.

2. Radiation-resistivity

The resistance to gamma-radiation of these isolates from sewage sludges[5] and animal feeds[6] was similar to that of *D. radiodurans* R_1 and other described species, and D_{10} values ranged from 0.10 to 0.25 Mrad in the aerobic irradiation conditions as shown in Table IV. However, the shape of survival curves was distinct for each strain, and many strains showed sigmoidal curves with different sized shoulders (Fig. 4). On the other hand, the shape of the survival curves of TD1 and *D. proteolyticus* CCM 2703 were exponential and this is not related to the morphology of diplococci or tetrads at the stationary phase of growth. The D_{10} values of each strain with the condition of air-equilibrium are almost the same as those with nitrogen gas equilibrium and this anaerobic condition could be attained after irradiation of more than 0.3 Mrad by consumption of oxygen. The oxygen enhancement ratio of all radiation-resistant cocci (D_{10} of anaerobic condition/D_{10} of air-bubbling) ranged from 1.0 to 1.5 as shown in Table IV, and these results show that the oxygen enhancement effect is not important for the radiation sensitivity of the strains of the genus *Deinococcus*. For example, the survival curve of TD1 obtained after irradiation with the condition of air-bubbling, air-equilibrium and nitrogen gas equilibrium showed the same D_{10} value and the same exponential curve, and these results are very distinct from other kinds of bacteria such as *Pseudomonas radiora*[13] and *Escherichia coli*.[15]

Catalase activity as shown by Kat. *f* for each strain was distributed from 2 to 212 indicating that the catalase activity is not related to the oxygen enhancement ratio and radiation-resistance for each strain of *Deinococcus*. This result is also very distinct from that of *P. radiora* in which catalase activity correlated with the oxygen effect as reported in the previous paper.[13]

3. Description

The detailed characteristics of the six isolates from sewage sludges and animal feeds are as follows.

Deinococcus proteolyticus Brooks and Murray 1981.

Strains: TD1, TD3, TD9, T843, Fr3, Fr7

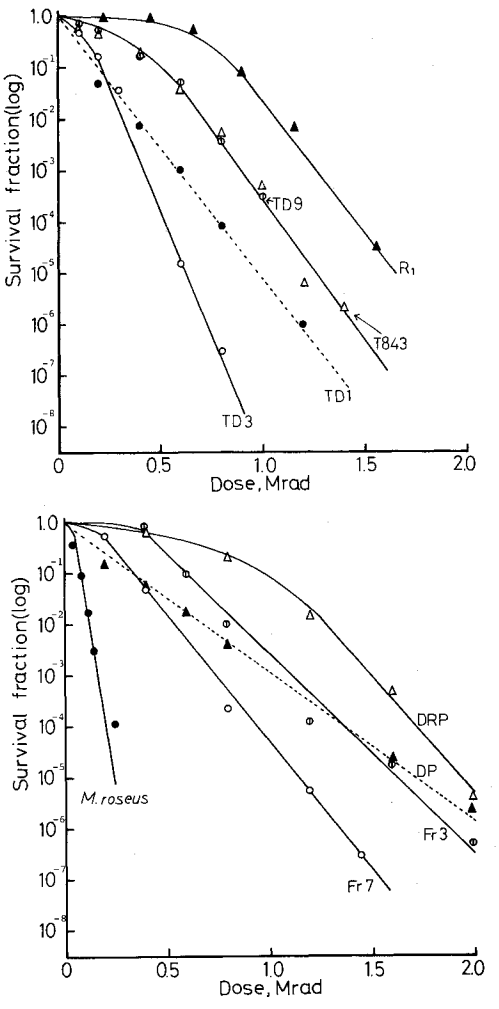

FIG. 4. Comparative Sensitivities of Several Strains of Radiation-resistant Micrococci to Gamma Irradiation under Air-bubbling in Phosphate Buffer.

R_1, *D. radiodurans* R_1; DRP, *D. radiophilus* CCM 2564; DP, *D. proteolyticus* CCM 2703.

Spheres, 0.8 to 1.0 μm in diameter, occurring singly or in pairs and sometimes dividing in two planes to form tetrads. Non-motile.

The peptide subunit of the cell wall contains ornithine. The predominant fatty acid component is $C_{16:1}$. No branched fatty acids are present.

Colonies on nutrient agar are generally orange to red and some strains are white to pink, smooth, and convex with a regular edge.

Chemoorganotrophic; metabolism is respiratory. Acid without gas is produced from glucose and sucrose when attacked. Casein and gelatin are hydrolyzed. Nitrates are reduced to nitrites or not reduced.

Strictly aerobic. Growth in the presence of 1% NaCl, but can not grow at 3% NaCl. Grow on nutrient agar without other growth factors.

Cytochrome oxidase and catalase positive.

The GC content of DNA is 59~66 mol% (by T_m).

Source: Sewage sludges and animal feeds after high dose gamma-ray irradiation.

DISCUSSION

Radiation-resistant cocci which belong to the genus *Deinococcus* recently proposed by Brooks and Murray[3] can be found in broad environments in Japan such as in sewage sludges,[5] mixed animal feeds,[6] and other sources[4,9] after irradiation at high doses. In this study, all isolates were able to grow on nutrient agar, and this characteristic was distinct from that of the type strain of *D. radiodurans*. One of the problems in defining *Deinococcus* species is that all the described strains were obtained with radiation as the selective factor, and there is a possibility of nutritional mutations occurring after irradiation. However, all of the isolates in this study seem to have wild type characteristics even after irradiation similar to other bacteria which can be identified as described species.[5,6,13] For this reason, it is supposed that the nutritional requirement is also important for taxonomical classification. Therefore, isolates TD1, TD3, TD9, T843, Fr3 and Fr7 were identified as *D. proteolyticus*. However, they had some different characteristics compared with type strain CCM 2703. Furthermore, strain T843 had different characteristics compared with the other five strains, and it is not considered to be a typical *D. proteolyticus*. In the case of isolates from sawdust culture media, H54 and H55 were confirmed to belong to *D. radiodurans* as in the previous report.[4] Whereas, H48 should be reidentified as *D. radiophilus* on the basis of its fastidious nutritional require-

ments and higher salt tolerance compared with other isolates. In this study, all *Deinococcus* strains showed radiation-resistance and a low oxygen enhancement effect, and these radiation effects are also important characteristics of the genus *Deinococcus*.

Further studies are needed on the taxonomic relation between the genera *Deinococcus* and *Micrococcus*, and among all *Deinococcus* isolates using methods such as DNA homology and interspecies transformation.

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