A Radioresistant Gram-positive Asporogenous Rod Isolated from the Faeces of a Giant Panda (*Ailuropoda melanoleuca*)

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SUMMARY

A highly radioresistant bacterium was isolated from the faeces of a giant panda (*Ailuropoda melanoleuca*). When the organism was subjected to gamma irradiation in phosphate buffer, the induction dose and D_{10} values were 846 and 345 krad, respectively, for cells grown on PCNZ agar, and 700 and 460 krad, respectively, for the enlarged cells grown on 5% (v/v) horse blood brain heart infusion agar. The D_{10} value of the former cells was about 1.8 times higher than that of *Micrococcus radiodurans* grown on PCNZ agar.

INTRODUCTION

Several bacteria which are highly resistant to gamma rays have been reported (Davis, Silverman, & Masurovsky, 1963; Ito & Iizuka, 1971; Lewis, 1971; Yoshinaka, Yano & Yamaguchi, 1973; Matsuyama *et al.*, 1973; Yano *et al.*, 1975; Welch & Maxcy, 1975) since the radioresistant bacterium *Micrococcus radiodurans* was first isolated from irradiated meat (Anderson *et al.*, 1956). Kobatake, Tanabe & Hasegawa (1973) reported radioresistant bacteria in the faeces of 30 kinds of animals, and isolated *Micrococcus radioproteolyticus* from the faeces of a llama (*Lama glama*). We report here the properties of a highly radioresistant Gram-positive asporogenous bacterium isolated from the faeces of the giant panda (*Ailuropoda melanoleuca*).

METHODS

Media. Three isolation media were used: nutrient agar (Difco); brain heart infusion (BHI) agar (Difco); and PCNZ agar (Krabbenhoft, Anderson & Elliker, 1967), which contained (g l⁻¹): tryptone, 5.0; yeast extract, 2.5; glucose, 1.0; NZ-case (Sheffield Chemical Co., Norwich, New York, U.S.A.), 5.0; agar, 15.0; pH 7.0.

Isolation of organisms. Faeces samples were collected from a healthy 4-year-old female and a 3-year-old male giant panda; they contained undigested bamboo leaves, sugar cane, jujube, chopped straw and mucus. Samples (1 ml) of dilutions of the faeces were spread on each of the plating media in stainless-steel trays (220 × 280 × 35 mm), and incubated at 37 °C for 1 week. Pure cultures were obtained by repeated subculture on PCNZ agar, and maintained on PCNZ agar slants. All strains were cultivated at 37 °C unless stated otherwise.

Identification methods. Morphological characters were recorded for strains grown on four different media: PCNZ agar; nutrient agar (Difco); 5% (v/v) horse blood BHI agar; and EYGA medium (Cure & Keddie, 1973), which contained (per l mineral base E): yeast extract, 1 g; glucose, 1 g; purified agar, 12 g; and vitamin B₁₂, 2 µg. Morphological changes during the growth cycle were examined in microcultures on EYGA medium, incubated at 25 °C, by the method of Komagata, Yamada & Ogawa (1969). Biochemical and physiological characters were tested as described by Yamada & Komagata (1972) and Cowan & Steel (1974), and vitamin requirements were examined by the method of Keddie, Leask & Grainger (1966). The principal amino acids in the cell walls and the GC content of the DNA were determined as described by Yamada & Komagata (1970*a, b*), and menaquinones isolated from lyophilized organisms were identified by paper chromatography and mass spectrometry (Yamada *et al.*, 1976).

For the toxicity test, 48 h cultures in BHI broth were centrifuged and 1 ml of the packed cells and 1 ml of the supernatant fluid were injected intravenously into each of five mice. Mean generation times were determined from growth curves by measuring the extinction of PCNZ broth cultures shaken in a Monod type L-tube.

Irradiation. Forty-six faecal isolates were screened for high radioresistance. 'Test pieces' for gamma irradiation (discs of dried bacteria), prepared by the method of Christensen & Holm (1964) as modified by Tanabe *et al.* (1971), were irradiated with doses ranging from 0.1 to 6.0 Mrad in rubber-stoppered test tubes (20 × 180 mm) containing about 5 g silica gel. Irradiation was carried out at a dose rate of 16.4 krad min⁻¹ at room temp. (about 18 °C) in a ⁶⁰Co plant (Institute of Physical and Chemical Research, Wako-shi, Saitama, Japan). Irradiated bacteria were suspended in 0.067 M-phosphate buffer pH 7.0 and serially diluted. Colony counts were made on PCNZ agar plates after 5 days incubation.

Strain P-30-A (the most radioresistant strain isolated), strain P-30-B (an orange-coloured mutant derived from strain P-30-A by a gamma radiation dose of 1.3 Mrad) and *M. radiodurans* R₁ (Duggan, Anderson & Elliker, 1963), which was kindly supplied by Dr Matsuyama, were irradiated in phosphate buffer to compare their radiosensitivities. The radiosensitivities of strain P-30-A grown under the same conditions on two different media were also compared since the shapes and sizes of these bacteria varied with the growth environment. Thus four types of cells were studied: single or paired cells of strain P-30-A grown on PCNZ agar (PCNZ-A cells); single or paired cells of strain P-30-B grown on PCNZ agar (PCNZ-B cells); enlarged and chained cells of strain P-30-A grown on the blood BHI agar (BHI-A cells); and cells of *M. radiodurans* R₁ grown on PCNZ agar (PCNZ-R₁ cells). The test strains, freshly subcultured on to PCNZ agar slants, were inoculated heavily on to PCNZ agar or blood BHI agar plates and incubated for 48 h. Phosphate buffer suspensions were then prepared to give a concentration of approximately 10⁸ cells ml⁻¹. BHI-A cells were carefully suspended to avoid damaging the chains. Test tubes containing 5 ml of the prepared suspensions were irradiated, with air equilibrium, at room temperature as before.

RESULTS

Isolation of organism

The number of aerobic bacteria found in the faeces of the two giant pandas was in the range 10⁷ to 10⁹ (g wet wt)⁻¹; the highest counts were obtained on PCNZ and blood BHI agar plates. Of the 46 bacterial strains isolated, which represented various colony types, 40 were relatively sensitive to gamma radiation (A, Fig. 1), whereas five strains, identified as *Bacillus cereus*, *B. subtilis*, *Micrococcus* sp., *Streptococcus* sp. and a coryneform bacterium,

Fig. 1

Fig. 2

Fig. 1. Survival curves for bacteria isolated from the faeces of the giant panda: area A, 40 unidentified strains; area B, *Bacillus cereus*, *B. subtilis*, *Micrococcus* sp., *Streptococcus* sp. and a coryneform P-2; ○, strain P-30-A. Cells grown on PCNZ agar were gamma-irradiated under dry conditions. The survival curve of *Corynebacterium equi* 1AM1038 (●) is included for comparison.

Fig. 2. Survival curves of strain P-30-A grown on PCNZ agar (○), strain P-30-B grown on PCNZ agar (●), strain P-30-A grown on blood BHI agar (△), and *M. radiodurans* R₁ grown on PCNZ agar (□). Cells grown at 37 °C for 48 h were gamma-irradiated in 0.067 M-phosphate buffer solution.

required doses of 2.0 to 3.7 Mrad to achieve an inactivation factor of 10^8 (B, Fig. 1). Strain P-30-A was the most radioresistant of the isolates; it was not exposed to gamma radiation in the course of its isolation.

Characteristics of strain P-30-A

Strain P-30-A was Gram-positive, aerobic, non-sporeforming, non-motile and produced rods which contained metachromatic granules. Coccoid cells, inoculated on to EYGA medium, elongated into rods forming a transverse septum by which fission took place. After 6 to 7 days coccoid cells appeared.

On PCNZ and nutrient agars, rods with rounded or tapered ends (0.7 to 0.9×0.9 to $3.0 \mu\text{m}$) occurred singly, in pairs or, rarely, as short chains when cultured for 6 h to 1 week; a few slightly curved, long filaments (0.8 to 1.0×40 to $60 \mu\text{m}$) or long cells were also found.

On blood BHI agar, slightly curved or V-shaped rods (0.8 to 1.0×1.2 to $3.0 \mu\text{m}$) were present after 6 to 8 h. After 24 h, chains and ghost-like spherical cells (1.0 to $5.0 \mu\text{m}$ diam.) formed. By 48 h the chains were 20 to $30 \mu\text{m}$ long and consisted of about 15 cells (1.5 to 2.5×1.5 to $3.0 \mu\text{m}$). Short chains, paired rods and swollen cells were also found. After 7 days large, long, translucent cells (1.0 to 2.5×10 to $70 \mu\text{m}$) containing small or large Gram-positive granules appeared.

On nutrient agar, circular, raised, smooth, entire, glistening, opaque, cream to pale flesh-coloured colonies were formed, and nutrient broth cultures were slightly turbid with little sediment.

The organism did not produce indole or H_2S , was methyl red and Voges-Proskauer negative, did not degrade cellulose or aesculin or change litmus milk, and did not produce acid from ribose, L-arabinose, xylose, rhamnose, glucose, fructose, mannose, galactose, sucrose, lactose, maltose, starch, glycerol, mannitol, sorbitol or inositol. Ammonia was not produced from arginine; oxidase and catalase were present; urease, lecithinase and tyrosinase were absent; an extracellular DNAase was detected; gelatin and starch were degraded; and nitrate was reduced to nitrite. Acetate, pyruvate, lactate, α -ketoglutarate, formate and

Table 1. *Radiosensitivity of the bacteria isolated from the faeces of the giant panda under dry conditions*

Organism	Dose ...	Surviving fraction		
		0.3 Mrad	1.0 Mrad	2.0 Mrad
Strain P-30-A		5.9×10^{-1}	1.8×10^{-1}	2.6×10^{-2}
<i>Micrococcus</i> sp. P-3		5.0×10^{-2}	8.9×10^{-5}	—
<i>Streptococcus</i> sp. P-43		8.3×10^{-2}	2.6×10^{-4}	1.0×10^{-7}
<i>Bacillus cereus</i> P-32		1.8×10^{-1}	5.3×10^{-3}	3.5×10^{-5}
<i>B. subtilis</i> P-10		2.0×10^{-1}	6.7×10^{-3}	5.1×10^{-5}
Coryneform P-2		1.2×10^{-1}	1.1×10^{-3}	1.6×10^{-6}
40 unidentified bacteria		$< 5.0 \times 10^{-2}$	$< 8.9 \times 10^{-5}$	—
<i>Corynebacterium equi</i> IAM1038		1.3×10^{-7}	—	—

Table 2. *Radiosensitivity of strain P-30-A grown on two different media, of its orange-coloured mutant P-30-B and of Micrococcus radiodurans R₁*

Organism	Induction dose (krad)	D ₁₀ value (krad)	Culture medium	Shape of cells at 48 h	Pigmentation of colony
Strain P-30-A	846	345	PCNZ	Single or paired rods	Cream to pale flesh
Strain P-30-B	855	334	PCNZ	Single or paired rods	Orange
Strain P-30-A	700	460	Blood BHI	Enlarged and chained rods	Greyish-cream
<i>M. radiodurans</i> R ₁	727	195	PCNZ	Single or tetrad cocci	Brownish-red

hippurate were utilized as sole sources of carbon for energy and growth but malate, succinate, fumarate, citrate, propionate, oxalate, gluconate and urate were not.

Neither the culture broth, nor its supernatant fluid after centrifuging, was fatal to mice.

No growth occurred in 5% (w/v) NaCl broth or in a mineral salts plus glucose medium unless vitamin-free Casamino acids (Difco) were added. The optimum temperature for growth was 37 °C; no growth occurred at 40 °C or below 10 °C. The organism was not resistant to heating at 63 °C for 30 min in skim milk. Its optimum pH was between 7.0 and 7.4.

The organism had a GC content of 65.7 mol %, contained lysine as the principal cell-wall amino acid, and possessed menaquinones with eight isoprene units (MK-8).

The organism cannot be classified with confidence at present, and has been deposited at the Institute of Applied Microbiology, University of Tokyo, Japan; accession number IAM12120.

The morphological, biochemical and physiological characteristics of strain P-30-B (IAM12140) are similar to those of strain P-30-A.

Radiosensitivities

Under dry conditions, the radiation resistance of strain P-30-A was considerably higher than the resistance of *Corynebacterium equi* IAM1038 and of a coryneform bacterium isolated from the faeces of a female giant panda (Fig. 1, Table 1). The shape of the survival curve of strain P-30-A resembled that of the coryneform bacteria illustrated by Christensen, Holm & Juul (1967).

In phosphate buffer strains P-30-A and P-30-B exhibited sigmoidal survival curves which were similar to that of *M. radiodurans* R₁ (Fig. 2). However, D₁₀ values on the exponential part of the survival curves for the two test strains were higher than that for *M. radiodurans* R₁.

D_{10} values of PCNZ-A and BHI-A cells were, respectively, about 1.8 and 2.4 times higher than that of PCNZ- R_1 cells. There was no correlation between pigmentation and radiation resistance (Fig. 2, Table 2) as investigated by Okazawa & Matsuyama (1967), and Suhadi *et al.* (1972).

DISCUSSION

Strains P-30-A and *M. radiodurans* R_1 showed approximately the same induction dose. Hence, if radiation induced similar lesions in their DNAs, their primary repair mechanisms must be qualitatively similar. However, there were large differences between the D_{10} values on the exponential part of the survival curves for these two bacteria. Kitayama & Matsuyama (1970) suggested that in *M. radiodurans* damage to cytoplasmic components, such as the system for protein synthesis, would become a critical factor in the survival of organisms subjected to high doses of irradiation. If this were so with strain P-30-A, then it is unlikely that the system for protein synthesis, which might be required for the completion of DNA repair, is damaged, otherwise the ability of strain P-30-A to recover from radiation damage would have to be greater than that of *M. radiodurans*.

There is little difference between the D_{10} values of PCNZ-A and BHI-A cells. In *Escherichia coli* the radiosensitivity of cells is affected by the composition of the culture medium and by the phase of the growth cycle (Stapleton & Fisher, 1967; Town, Smith & Kaplan, 1970). The difference in D_{10} values between PCNZ-A and BHI-A cells could, therefore, be due to differences in DNA repair ability or protein synthesis activity which reflect the different growth media.

Strain P-30-A not only shows considerable radiation resistance but also has a short mean generation time (60 min), and it is not difficult to extract DNA from the organism. This bacterium might, therefore, be suitable for investigations into the mechanism of extreme radiation resistance in bacteria.

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