Studies on a Radio-resistant Coccus Isolated from Bombay Duck (*Harpodon nehereus*)

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SUMMARY

An extremely radio-resistant orange-red pigmented *Micrococcus* sp. was isolated from irradiated Bombay duck (*Harpodon nehereus*). The organism was a Gram-positive vegetative tetracoccus, catalase-positive, sensitive to heat and non-pathogenic. It differed from *M. radiodurans* by being smaller in size, salt-tolerant, hydrolysing gelatin very slowly, and much more resistant to gamma radiation. The radiation survival curve of the new coccus was sigmoidal, punctuated with a very large shoulder extending beyond 1·5 Mrad.

INTRODUCTION

Micrococcus radiodurans, isolated originally by Anderson *et al.* (1956) from meat exposed to 4 Mrad, has evoked considerable attention because of its unique radio resistance. Ever since its isolation this organism has served as a dynamic model for studies in mechanisms of radio resistance in bacteria. The radio resistance of *M. radiodurans* has been postulated to be due to pigmentation (Moseley, 1963; Krabbenhoft, Anderson & Elliker, 1967), the presence of mercaptoalkylamine (Anderson *et al.* 1959), intracellular radio-protective compounds (Bruce, 1964), the ultrafine structure of the cell wall (Thornley, Horne & Glauert, 1965) and to fatty-acid composition (Lewis, 1965). However, the work of Setlow & Duggan (1964) and more recently of Moseley & Laser (1965) implies that the high radio resistance of *M. radiodurans* may be attributed to enzyme repair mechanisms for damaged deoxyribonucleic acid (DNA). *Micrococcus radiodurans* has been subsequently isolated from other sources (Krabbenhoft, Anderson & Elliker, 1965). Two other radio-resistant *Micrococcus* species, morphologically similar to *M. radiodurans*, have since been reported – from irradiated haddock fillets (Davis, Silverman & Masurovsky, 1963) and an aerial contaminant (Murray & Robinow, 1958).

It was therefore of interest to investigate if this organism was universally present; and, if so, whether it occurred in irradiated tropical fish varieties. During investigations on the extension of the shelf-life of Bombay duck (*Harpodon nehereus*) using gamma radiation, glistening orange-red pigmented colonies in Petri dish cultures were consistently isolated from fillets exposed to 1·0 to 2·0 Mrad. When this organism was examined for radiation resistance, cultural, morphological and biochemical characteristics, it was observed that the isolate was distinct from *Micrococcus radiodurans* or any other *Micrococcus* species listed in *Bergey's Manual*, suggesting that it might be a new species. Pure cultures were found to be more resistant to gamma radiation than any microbial species so far reported. The newly isolated coccus was designated RBD, denoting a radio-resistant micro-organism obtained from Bombay duck.

This culture has been deposited with the National Collection of Type Cultures, Colindale, London.

METHODS

Cultures. *Micrococcus radiodurans*, obtained from Professor A. W. Anderson, Oregon State University, Oregon, U.S.A., which was used for comparative purposes throughout this investigation, was cultured in TGYM medium containing 0.5 % tryptone, 0.1 % glucose, 0.3 % yeast extract and 50 mg. % DL-methionine.

Microbial evaluation of Bombay-duck fillets subjected to 2.0 Mrad indicated that only orange-red pigmented cocci survived the radiation treatment. The original isolate was cultured on nutrient agar and subsequently maintained on TGYM agar slants.

Growth characteristics

Effect of media on growth stimulation was investigated with nutrient agar, TGYM (tryptone, 0.5 %; glucose, 0.1 %; yeast extract, 0.5 %), tryptone glucose extract (TGE) agar, as well as TGY agar incorporated with DL-methionine (50 mg. %) or Bacto Casamino acids (0.5 %). Growth was evaluated by streaking the surface of deep agar slants and observing periodically over a 6 day incubation period at room temperature (28°). The effect of DL-methionine (essential for propagation of *Micrococcus radiodurans*) on growth was investigated by incorporating varying concentrations from 20 mg. to 2 g./l. of TGY broth. The effect of pH on growth was ascertained by culturing the organisms in TGYM broth at pH levels from 5.5 to 9.5. Tolerance of NaCl up to a concentration of 7 % in TGYM broth was also investigated. For optimal growth-temperature experiments, inoculated slants were incubated at 15°, 28°, 37° and 45° and growth was assessed by visual examination.

Biochemical tests. Several biochemical tests (Harrigan & McCance, 1966) were done, including catalase formation, hydrogen sulphide production, gelatin liquefaction, indole production, nitrate reduction, and both acid and gas production from carbohydrates. The inocula for all tests were taken from 24 h. TGYM broth cultures.

The catalase test was performed by adding 1 ml. culture to 1 ml. of 10 vol. H₂O₂ and looking for effervescence within 5 min.; H₂S production was demonstrated on triple sugar iron agar slants; gelatin liquefaction was assessed with nutrient gelatin stabs incubated for 30 days at 25°; indole production in 1 % tryptone solution was detected by Kovac's indole reagent. Reduction of nitrate was demonstrated after maximal growth was attained (72 h.). The basal medium for fermentation of carbohydrates was 1 % peptone water incorporating Andrade's indicator for detection of acid produced. Sugars were heat-sterilized as 10 % solutions and used at a final concentration of 1 %. Morphological characteristics were studied by taking a loopful of culture on a coverslip, drying in air, inverting over a drop of lactophenol on a glass slide and examining under phase contrast.

Toxin assay. Twenty-four h. and 7 day TGYM broth cultures were examined for pathogenicity. After centrifugation 1 ml. samples of supernatants and sonicated cell suspensions were injected intraperitoneally into mice which were observed for 8 days. Six mice were used for each sample.

Irradiation

The radio-resistant coccus RBD and *Micrococcus radiodurans* were cultured for 40 to 44 h. at room temperature, in shake flasks containing TGYM broth (pH 7.2). The populations were then standardized to a density of approximately 10⁸ bacteria/ml. by extinction measure-

ments, and 5 ml. suspensions were dispensed in 9×1 cm. test tubes, placed in a beaker containing comminuted ice and irradiated within 30 min. in a Gamma-cell 220 (A. E. C. L., Canada) at a dose rate of 11 Krad/min. Temperature during irradiation was maintained at 4° to 6° . Radiation survivors were recovered on TGYM agar. Colonies were counted after 5 days incubation at room temperature.

RESULTS

By phase-contrast microscopy the new isolate was seen to be a *Micrococcus* species morphologically similar to *Micrococcus radiodurans* (Pl. 1, fig. 1, 2). The organisms occurred predominantly as tetrads, and occasionally in pairs or singly. However, the organism was smaller than *M. radiodurans*, being spheres averaging $1.5 \pm 0.2 \mu\text{m}$. in diameter as compared with $2.4 \pm 0.46 \mu\text{m}$. for *M. radiodurans*. Microscopic examination of cultures at different physiological ages did not reveal any over-size cells; *M. radiodurans* cultures normally contained a small percentage of very large cells.

The new coccus always stained Gram-positive, unlike older cultures of *Micrococcus radiodurans*, which were reported as Gram-variable (Anderson *et al.* 1959). Growth obtained on TGYM agar slants after 72 h. incubation at 30° was mucoid, raised glistening orange-red as compared with dull-red growth of *M. radiodurans*. Moreover, unlike *M. radiodurans* the pigment did not diffuse into the agar. Growth rate and pigmentation were greater on TGY agar than on TGE agar where the growth was poor, or on nutrient agar which barely supported growth. TGY agar with DL-methionine (50 mg. %) or supplemented with 0.5 % Casamino acids (acid-hydrolysed casein) did not change growth or pigmentation. Krabbenhoft *et al.* (1967) reported greater yields but less pigmentation in cells of *M. radiodurans* grown in TGY medium supplemented with 0.5 % tryptic digest of casein (NZ-case). Use of deionized water for media preparation resulted in poor growth as compared with tap water where both growth rate and yield were considerably enhanced, indicating trace-metal requirement in growth media. Growth was greatly stimulated by vigorous aeration. DL-Methionine, said to be an essential amino acid for growth of *M. radiodurans* (Raj *et al.* 1960), did not enhance growth of RBD up to a level of 400 mg./l.; higher concentrations reduced the yield.

Table 1 indicates the pronounced differences produced in the growth characteristics of *Micrococcus radiodurans* and RBD by altering the pH of the growth media: *M. radiodurans* had optimum growth at acid pH (5.5 to 6.5) and grew well up to pH 8.5, above which growth was markedly inhibited, while RBD did not grow at pH 6.0 or below but grew well up to pH 9.5. The salt tolerance of these two micro-organisms is recorded in Table 2. The isolate RBD grew well in media containing NaCl up to 3 % and moderately in media containing 5 % NaCl; *M. radiodurans* was inhibited at 2 % NaCl. Temperature affected both organisms similarly. The optimum was 30° to 37° , while growth was slow at 20° and nil at both 10° and 45° .

Both *Micrococcus radiodurans* and RBD possessed high catalase activity but did not reduce nitrate nor produce indole from tryptophan. While *M. radiodurans* liquefied gelatin within 48 h., no liquefaction was obtained with RBD cultures up to 24 days incubation at 25° ; after this, liquefaction proceeded at a very slow rate. Anderson *et al.* (1956) reported that samples of beef seeded with *M. radiodurans* underwent spoilage which was characterized by considerable proteolysis and CO_2 production. However, no proteolysis or gas production could be detected in sterile fish homogenates inoculated with RBD cells and incubated for 48 h. at 30° . The results of the carbohydrate fermentation reactions are listed in Table 3. The new isolate readily produced acid from glucose, fructose and mannitol and gave variable results with

lactose and galactose; *M. radiodurans* did not produce acid from any of the carbohydrates used. Neither organism produced gas from any of the carbohydrates examined.

Results of the toxin assay indicated that both *Micrococcus radiodurans* and RBD were non-pathogenic.

A comparison of the radiation resistivity of RBD and *Micrococcus radiodurans* is presented in Fig. 1. The survival curve of RBD is similar to that of *M. radiodurans* in being sigmoidal but is easily distinguished by a very large shoulder extending beyond 1.5 Mrad. A radiation dose of 1.6 Mrad, which sufficed to reduce the viable cell population of *M. radiodurans* to about one-millionth, resulted in only tenfold inactivation of RBD cell suspensions. A dose of 3.2 Mrad was necessary to reduce the viable cell count of RBD to a millionth.

Table 1. *Effect of pH on growth of Micrococcus radiodurans and RBD*

pH of growth media	Growth* after 48 h.	
	<i>M. radiodurans</i>	RBD
5.5	+++++	—
6.0	+++++	—
6.5	+++++	+++
7.0	++++	+++++
7.5	++++	+++++
8.0	+++	++++
8.5	+++	++++
9.0	+	+++
9.5	—	+++

* +++++, Excellent; +++++, very good; +++, good; +, scanty; —, nil.

Table 2. *Salt tolerance of Micrococcus radiodurans and RBD*

NaCl concentration in growth media	Growth* after 48 h.	
	<i>M. radiodurans</i>	RBD
0%	+++++	+++++
1%	++++	+++++
2%	—	+++++
3%	—	+++++
5%	—	+++
7%	—	—

* Symbols as in Table 1.

Table 3. *Production of acid and gas during carbohydrate utilization by Micrococcus radiodurans and RBD*

Carbohydrate (1 %)	<i>M. radiodurans</i>		RBD	
	Acid	Gas	Acid	Gas
Arabinose	—	—	—	—
Fructose	+	—	—	—
Galactose	Var	—	—	—
Glucose	+	—	—	—
Glycerol	—	—	—	—
Inositol	—	—	—	—
Lactose	Var	—	—	—
Mannitol	+	—	—	—
Rhamnose	—	—	—	—
Sucrose	—	—	—	—
Xylose	—	—	—	—

—, Negative result; +, positive result; Var, variable result.

Fig. 1. Percentages of RBD (▲) and *Micrococcus radiodurans* (■) surviving different doses of gamma radiation.

DISCUSSION

Preliminary studies on cellular components of the new *Micrococcus* species, which is more resistant to gamma radiation than *Micrococcus radiodurans* by several orders of magnitude, indicate that besides the evident possession of an extremely efficient enzyme repair system, other factors like pigments and lipids could presumably have contributed to its exceptional radio resistance. The isolation of a new radio-resistant *Micrococcus* sp. provides further impetus towards establishing mechanisms that may clarify the unique radio resistivity of these organisms.

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Fig. 1

Fig. 2

PLATE I

Phase-contrast photomicrographs of *Micrococcus radiodurans* (Fig. 1) and RBD (Fig. 2) in shake cultures.