RADIATION-RESISTANT, PIGMENTED COCCUS ISOLATED FROM HADDOCK TISSUE[1]

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ABSTRACT

DAVIS, NORMAN S. (Massachusetts Institute of Technology, Cambridge), GERALD J. SILVERMAN, AND EDMUND B. MASUROVSKY. Radiation-resistant, pigmented coccus isolated from haddock tissue. J. Bacteriol. **86**:294–298. 1963.—An orange-brown, catalase-positive coccus was isolated from irradiated haddock. The new coccus was found to consist of rough and smooth strains as well as strains possessing appreciably less pigment. The smooth strain (248) was more radioresistant to gamma radiation than the rough strain, and at higher radiation doses was of comparable resistance to *Micrococcus radiodurans*. The less-pigmented strain was as radioresistant as its parent strain (248). Although morphologically similar to other, but less-resistant, micrococci, the coccus differed by growing slowly on solid media, and was unable to grow in 7% salt. It was distinguished from *M. radiodurans* by being smaller in size and in being capable of reducing nitrate to nitrite and hydrolyzing gelatin.

During the course of an investigation on the effects of gamma rays upon the microflora of haddock, we consistently noted colonies of orange-brown pigmented cocci on petri dish cultures from fillets of haddock treated with doses of from 50,000 to 800,000 rad of ionizing radiation. Exposure of pure cultures of the isolates to gamma radiation revealed them to be a very resistant vegetative species.

Anderson et al. (1956) previously isolated a red-pigmented radiation-resistant micrococcus, designated *Micrococcus radiodurans*, from meat exposed to 2 to 3 megarad of gamma rays. Murray and Robinow (1958) also isolated a large radiation-resistant air contaminant consisting of four daughter cells produced by simultaneous division, a feature shared by *M. radiodurans*. Since these are the only two vegetative organisms reported to date which are extremely resistant to radiation, it is of interest to report a third, derived from a different source and morphologically distinct from *M. radiodurans*.

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MATERIALS AND METHODS

The original isolate from haddock homogenates grew on Eugonagar supplemented with 0.5% yeast extract. Subsequent studies indicated that growth was superior on Plate Count (PC) agar, which was supplemented with 0.5% N-Z-case (NZ; Sheffield Chemical Co., Norwich, N.Y.; also used for maintaining *M. radiodurans*), in comparison with Veal Infusion Agar, nutrient agar, PC agar without supplementation, Brain Heart Infusion Agar, and Tryptone Glucose Extract Agar. Growth was evaluated by streaking the surface of deep agar slants followed by stabbing the inoculum to the bottom of the tube. The cultures were held at room temperature and observed periodically over a 7-day period.

The original culture was found to consist of smooth and rough variants which were designated cultures 248 and 249, respectively, and a smooth, light-pink variant (253).

The radiation-resistant cultures 248, 249, and *M. radiodurans* were grown in shake flasks containing PCNZ broth for 36 to 40 hr at 30 C, harvested by centrifugation, and washed three times in 0.022 M phosphate buffer (pH 7.0). The cell population was then standardized to a bacterial density of approximately 10^8 cells/ml by optical density measurements, and 4 ml of cell suspension were syringed into 5-ml ampules and sparged with air for 15 min before the ampules were heat-sealed. Irradiation was conducted in a submerged pool-type Co^{60} source with a dose rate of 5,500 rad/min. The contents

of each ampule were serially diluted and plated in PCNZ agar. Colony counts were made after 5 days of incubation at 30 C.

For comparative purposes, the radioresistance of three strains of *M. roseus* (ATCC 179, 185, and 416) and *M. rubens* ATCC 186 was determined, in addition to strains 248, 249, and *M. radiodurans*. In these experiments, where cells were irradiated in their growth media (PC broth plus NZ), 3-ml portions of a 36- to 40-hr shake culture were placed directly into 5-ml ampules, sealed, and irradiated without air sparging.

For optimal growth-temperature experiments, inoculated slants were placed at temperatures of 20, 25, 30, 37, and 45 C, and growth was estimated visually. Gelatin liquefaction was determined in PCNZ with 4% gelatin instead of agar. Tolerance to salt was evaluated by streaking prepoured agar plates containing salt levels of 0, 1, 2, 3, 5, and 7%.

For phase-contrast microscopy, one loopful of a broth culture was dried on a cover slip, inverted, and placed on a thin agar layer on a microscope slide.

Results and Discussion

A comparison of the radiation resistivity of the washed smooth form (248), a rough form (249), and *M. radiodurans* is presented in Fig. 1. The rough form, although possessing high resistivity for a vegetative cell, was considerably less resistant than the smooth strain 248. The nature of the survival curves of the new isolates differed from that of *M. radiodurans* by not being sigmoidal, 248 being exponential and 249 resembling the type B curve of Gunter and Kohn (1956). At doses in excess of 1 megarad, the resistivity of strain 248 was equal to or exceeded that of *M. radiodurans*. Although the radioresistivity for *M. radiodurans* shown here is less than that described by Anderson et al. (1956) or Duggan et al. (1959), the techniques in our experiments differed in that washed cells were suspended in buffer and irradiated as such, whereas in the other studies washed cells were placed in broth and frozen prior to and subsequent to gamma-ray exposure. The radioresistivity of the R_1 strain, designated by Kilburn, Bellamy, and Terni (1958) as a *Sarcina* sp. but obtained from Anderson as *M. radiodurans* (R_1), had a D_{37} of up to 0.3 megarad in phosphate buffer. Berk (*unpublished data*) noted that 99% inactivation of *M. radiodurans* occurred in the range of 1.4 to 2.0 megarad. The new smooth strain (248) tested in this study possessed a D_{37} of 0.58 megarad in air and a D_{99} of 1.1 megarad.

Both strain 248 and *M. radiodurans* are distinguished from morphologically related micrococci by a greater radioresistivity (Table 1). At 0.1 megarad there was little difference in survival. In contrast, after exposure to 0.5 megarad, strain 248 and *M. radiodurans* were at least three orders of magnitude more resistant than the most resistant strain of *M. roseus* examined. Reference to Fig. 1 shows that the resistivity of cells suspended in broth was greater than washed cells. The final pH of the shake culture was not a factor in influencing radioresistivity. The lowest pH noted occurred in the most resistant of the *M. roseus* cultures. The pH of the other three micrococci cultures was comparable to that found in the flasks of strain 248 and *M. radiodurans*. The four micrococci, in addition to strains 248, 249, and *M. radiodurans*,

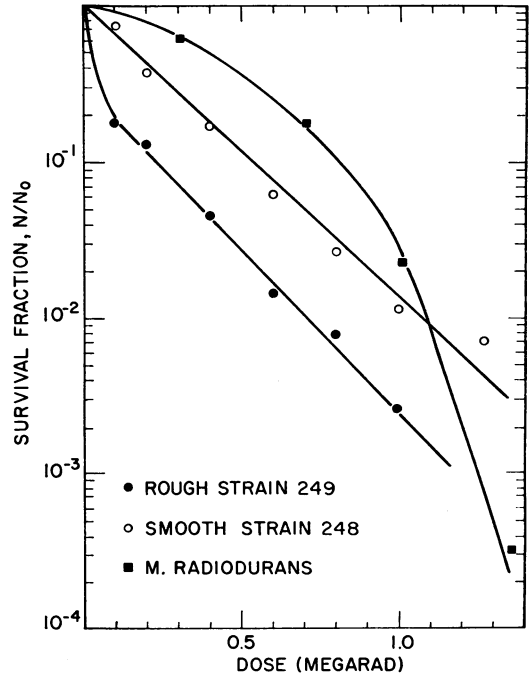

FIG. 1. *Radiosurvival of washed cells of the rough and smooth variants of newly isolated coccus and Micrococcus radiodurans.*

TABLE 1. *Radiation resistivities of shake cultures of the smooth strain of new isolate, 248, morphologically related micrococci, and Micrococcus radiodurans*

Culture	Survival fraction		Final pH of shake culture
	100,000 rad	500,000 rad	
M. roseus ATCC 179	1.0×10^{-1}	3.1×10^{-8}	8.6
M. roseus ATCC 185	8.3×10^{-1}	4.2×10^{-7}	8.6
M. roseus ATCC 416	5.8×10^{-1}	1.2×10^{-4}	7.1
M. rubens ATCC 186	5.7×10^{-1}	3.2×10^{-8}	8.4
Strain 248	8.0×10^{-1}	2.3×10^{-1}	8.2
M. radiodurans	8.2×10^{-1}	4.8×10^{-1}	8.4

did not produce acid or H_2S when inoculated into Triple Sugar Iron Agar.

The smooth, less-pigmented isolate (253) was found to be of comparable resistance to the fully pigmented, smooth strain (248) form from which it was isolated. Berk (*unpublished data*; Niven, 1958) previously noted for *M. radiodurans* that the absence of pigmentation, obtained by culturing this organism under reduced oxygen tension, did not alter radiation resistivity. In contrast, Kilburn et al. (1958) did note a reduction in resistivity for these cells when grown under reduced oxygen tension.

The rough and smooth strains were morphologically different from the large cells of *M. radiodurans*, and were comparable in size to micrococci (Fig. 2). The smooth strain tended to form diplococci and some tetrads, whereas the rough strain formed packets. A small number of very large cells were present in both cultures.

Niven (1958), describing the work of Berk, stated that the shape of the sigmoidal survival curve of *M. radiodurans* may be due to the formation of packets. Both 248 and 249 displayed exponential curves, and yet strain 248, the smooth form, although consisting mostly of pairs, was the more resistant of the two. It would thus appear that clustering had little effect on inherent radioresistivity and that an appreciable portion of the population was of comparable resistance to *M. radiodurans*.

The original isolate consisted of catalase-positive, gram-positive to gram-variable cocci. The growth on slants at 30 C was filiform, raised, and orange-brown. Unlike *M. radiodurans*, the pigment did not readily diffuse into the agar.

Strains 248 and 249 displayed different growth patterns on or within the various media chosen. As regards growth with respect to oxygen, it was noted that for strains 248 and 249 certain of the media (PC agar, Tryptone Glucose Extract Agar, and nutrient agar) which did not support extensive surface growth nevertheless did show growth extending to the bottom of the stab. On PCNZ agar, Brain Heart Infusion, and Eugonagar appreciable surface growth occurred, but growth in the stab was not observed below one-half of the agar depth. In contrast, copious growth was evident in Veal Infusion Agar on both the surface streak and the entire length of the agar stab. PCNZ agar or broth was the medium of choice for propagation. The optimal growth range was 25 to 30 C, cell multiplication being much less at 20, 37, and almost nonexistent at 45 C. An inability to grow in 2% sodium chloride media was distinctive for *M. radiodurans* (Anderson et al., 1956). Strains 248 and 249 grew well in 2% salt, moderately in 3% salt, scantily in 5% salt, and not at all in 7% salt. The other micrococci tested (Table 1) all grew profusely on agar containing 7% salt. The isolates also differed from *M. radiodurans* in being able to reduce nitrate and hydrolyze gelatin.

The light-pink variant differed in a number of ways from strains 248 and 249. Growth was considerably slower, it did not grow at 37 C and appeared to grow best at 25 C. It was almost completely inhibited by 3% salt. Morphologically, it was identical to strain 248, and the presence of large cells was noted.

The extremely high radioresistivity of *M. radiodurans*, a heat-sensitive vegetative organism, has been postulated as being due to pigmentation (Kilburn et al., 1958), to the presence of mercaptoalkylamine (Anderson et al., 1959), or to multicellularity (Stapleton, 1960). Murray (1962), employing electron microscopy, has shown that both *M. radiodurans* and an airborne apparently identical radiation-resistant organism, which was isolated in his laboratory, have a unique surface structure not as yet found elsewhere. The new species is being examined by Dr. Murray to determine its relationship to other cocci.

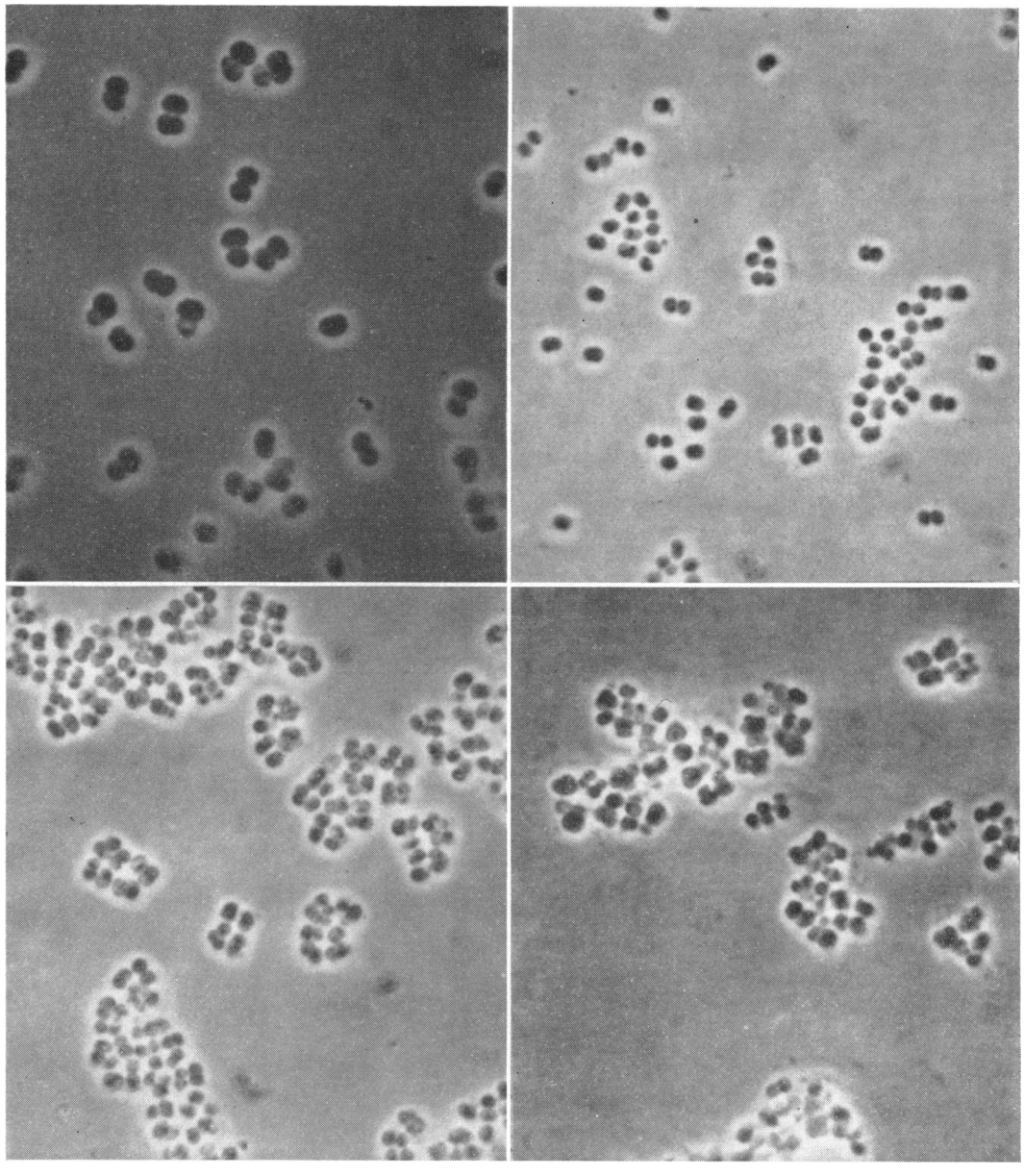

FIG. 2. *Phase-contrast photomicrographs of shake cultures of Micrococcus radiodurans (upper left), smooth coccus 248 (upper right), rough variant 249 (lower left), and M. roseus ATCC 416 (lower right).*

The isolation of a new radiation-resistant organism tempts one to postulate that other radioresistant organisms may exist. The fact that this coccus differs from *M. radiodurans* presents the opportunity to find any disparate or similar mechanisms that may clarify the unique radioresistivity of these vegetative species.

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