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Isolation of Radiation-Resistant Bacteria Without Exposure to Irradiation†

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Resistance to desiccation was utilized in the selection of highly radiation-resistant asporogenous bacteria from non-irradiated sources. A bacterial suspension in phosphate buffer was dried in a thin film at 25°C and 33% relative humidity. Storage under these conditions for 15 days or more reduced the number of radiation-sensitive bacteria. Further selection for radiation-resistant bacteria was obtained by irradiation of bacteria on velveteen in the replication process, thereby avoiding the toxic effect of irradiated media. The similarity of radiation resistance and identifying characteristics in irradiated and non-irradiated isolates should allay some concerns that highly radiation-resistant bacteria have been permanently altered by radiation selection.

Radiation-resistant bacteria have been isolated from various sources as the survivors of high doses of ionizing radiation (1, 8, 11, 20). The characteristics of these bacteria have been widely studied in order to assess their taxonomic positions and significance as part of the residual flora of irradiated foods.

Considering the highly mutagenic action of ionizing radiation (18), concern has been expressed that these bacteria may have been altered by the isolation procedure (8, 16). Permanent changes in bacteria after one or more doses of radiation have included increases in radiation resistance (3, 5, 12, 14), increases in antibiotic resistance (15), morphological changes (5, 12), biochemical changes (5), and decreased pathogenicity (6, 10, 15, 19).

A procedure designed to isolate radiation-resistant bacteria which have never been irradiated would more likely result in isolates whose characteristics correspond to those of naturally occurring food contaminants. Such a procedure would ideally be selective for or enrich the numbers of radiation-resistant pink micrococci (1, 11) as well as the resistant *Moraxella-Acinetobacter* (M-A) types studied by Welch and Maxcy (20).

Attempts to develop a method for isolation of radiation-resistant bacteria without the use of irradiation have been unsuccessful (16; A. W. Gulistani, Ph.D. thesis, University of Nebraska, Lincoln, 1977). Although Anderson et al. (1) isolated *Micrococcus radiodurans* from non-irradiated as well as irradiated beef, the isolation of radiation-resistant bacteria from non-irradiated sources was limited to the pink to orange-red micrococci. These highly pigmented radiation-resistant bacteria comprise a very low relative percentage of the total bacterial number (1, 13), making their isolation from non-irradiated sources difficult.

Dimmick and Akers (4) reported that *M. radiodurans* was very resistant to aerosol desiccation. Preliminary evidence indicated that the radiation-resistant M-A were also very resistant to desiccation (Gulistani, Ph.D. thesis). Based on this evidence, the factor of resistance to desiccation was included in a selective procedure followed by replicate plating and irradiation for isolation of non-irradiated resistant bacteria.

MATERIALS AND METHODS

Cultures and desiccation-selection. Known cultures of highly radiation-resistant M-A (20) were grown in m-Plate Count Broth (PCB; Difco) at 32°C for 24 h in a shaker incubator. Samples (0.01 ml) were spread onto sterile 1-cm^2 pieces of stainless steel and stored in a sterile environment at 33% relative humidity maintained by a saturated magnesium chloride solution at 25°C (21). The fate of these cultures was monitored by the method of Barnhart et al. (2).

Cattle hair, a rich source of radiation-resistant bacteria (Gulistani, Ph.D. thesis), was obtained from beef cattle at the time of slaughter. A bacterial suspension was obtained by shaking 1 g of hair in 99 ml of sterile phosphate buffer (7) for 3 min. After overnight storage at 25°C, 0.01-ml samples were spread onto stainless steel plates and studied as described above.

Ground beef was obtained from a local supermarket. Samples (0.01 ml) from a 1:10 dilution were spread on the steel plates and studied as described above.

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Isolation of radiation-resistant bacteria. The cattle hair or ground beef microflora, dried on stainless steel for 14 days to 5 months, was dislodged into phosphate buffer and serially diluted to yield approximately 100 colonies when spread on Plate Count Agar (PCA; Difco). From each sample, 12 PCA plates were spread with 0.1 ml each of the suspension and incubated at 32°C. After incubation, a replicate of each plate was made by using a velveteen replicator, after which the replicators were irradiated. Doses up to 800 Krads were provided by a cobalt 60 source at a dose rate of approximately 8 Krads/min. After irradiation, another set of imprints was made. Bacteria which grew on the plates derived from the irradiated replicators were presumptively radiation resistant. Their positions were matched with those of the non-irradiated replicators, thus allowing isolation of presumptively radiation-resistant bacteria which had not undergone irradiation. Corresponding irradiated and non-irradiated colonies were picked and studied further to confirm their common origin. The isolates were propagated on PCA slants.

To determine radiation resistance, the isolates were grown in PCB at 32°C for 24 h on a shaker incubator; 5-ml portions were frozen and irradiated at −30°C.

The bacteria were placed into groups based on the scheme of Welch and Maxcy (20). Radiation death curves were determined for some of the most resistant isolates after preliminary screening on the basis of resistance to 1 Mrad.

RESULTS

Selection through desiccation. Highly radiation-resistant M-A isolates 4, 7, and 13 were found to be very resistant to desiccation (Fig. 1). Their numbers after 166 days had decreased only approximately 1 log cycle. The general microflora of ground beef and cattle hair, however, showed a decrease of approximately 2 to 3 log cycles with comparable treatment (Fig. 1). The major part of the reduction in total numbers occurred during the first 7 to 15 days. The surviving microflora after 15 days consisted of four main groups: M-A, pink micrococci, *Micrococcus* spp., and *Bacillus* spp. (Table 1).

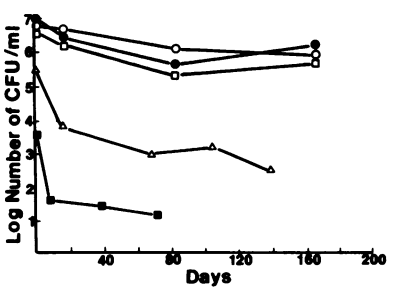

FIG. 1. *Fate of M-A, cattle hair flora, and ground beef flora during desiccation at 33% relative humidity. Symbols:* ○, *M-A 4;* ●, *M-A 7;* □, *M-A 13;* △, *cattle hair;* ■, *ground beef. CFU, Colony-forming units.*

The majority of the pink micrococci were very slow in recovering from desiccation, not forming visible colonies on subsequent plating and incubation until 4 to 5 days. The incubation time for master plates to be subsequently replicated, however, was limited to 24 h for cattle hair due to the common occurrence of rapidly spreading spores. For ground beef, the presence of fewer spores allowed a 48-h incubation. The identity of pink micrococci was apparent for selection purposes due to their distinctive pigmentation.

Desiccation-resistant bacteria surviving irradiation. Conventional methods of isolating bacterial variants using replicate plating have involved the treatment of one replicate with the agent of selection (i.e., radiation). This method proved unsatisfactory, as irradiated PCA was very toxic to radiation-resistant M-A. The problem was alleviated by irradiating the velveteen replicators rather than the PCA plates.

Bacteria surviving 400 to 800 Krads were limited to the M-A and a few pink micrococci. Irradiation of the replicators with 400, 600, and 800 Krads resulted in approximately 50, 98, and 99% reductions in bacterial numbers, respectively. Randomly selected colonies from the 400-Krad dose level showed a wide range of resistance to 1 Mrad, as indicated by surviving fractions of 4.0×10^{-7} to 0.66. Therefore, it was apparent that radiation-sensitive as well as radiation-resistant M-A survived the desiccation treatment. Isolate B-3, a pink micrococcus, was the most radiation-resistant isolate from beef at the 400-Krad dose level, but it was considerably less resistant than M-A 7 (Fig. 2).

All of the isolates from the 600- and 800-Krad dose levels were tested for radiation resistance. Only three showed a population reduction of more than 1 log cycle by a 1-Mrad dose. Isolate H-30 from cattle hair was comparable in radiation resistance to M-A 7. Each showed a large shoulder in the death curve extending to approximately 4 Mrads (Fig. 2). Other isolates, such as H-21, showed smaller shoulders and

TABLE 1. *Approximate distribution of bacterial groups from ground beef and cattle hair surviving 15 days of desiccation at 33% relative humidity, expressed as percentage of the total flora*

Bacteria	% in:	
	Cattle hair	Ground beef
M-A	35	42
Pink micrococci	23	15
Other micrococci	22	38
Bacillus	19	5

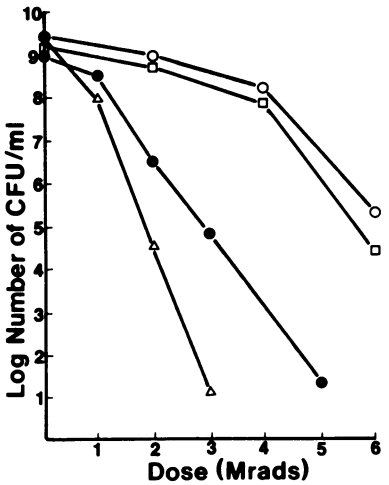

FIG. 2. *Radiation death curves of H-21, H-30, B-3, and M-A 7 in frozen Plate Count Broth. Symbols: △, H-21; □, H-30; ●, B-3; ○, M-A 7. CFU, Colony-forming units.*

were considered to have intermediate radiation resistance (Fig. 2).

Identifying characteristics. All of the isolates from the 600- and 800-Krad dose levels were gram-negative to gram-variable, nonmotile cocci or coccobacilli occurring primarily in pairs with adjacent sides flattened. Some isolates formed short chains or occasionally tetrads. All were oxidase and catalase positive. They did not oxidize or ferment glucose. They grew, but produced no apparent reaction in litmus milk. These isolates showed the same morphological and physiological characteristics as those obtained from beef by Welch and Maxcy (20) through selection by irradiation.

DISCUSSION

The results indicated that radiation-resistant M-A and pink micrococci are also very resistant to desiccation. Therefore, the combination of selection by desiccation and the replicate plating method allowed the isolation of radiation-resistant bacteria that had not been irradiated.

The availability of radiation-resistant bacteria that had not been exposed to the mutagenic effects of radiation allowed comparison with isolates obtained by radiation selection. The isolates obtained in this study, although not exposed to ionizing radiation, showed the same range of radiation resistance and the same identifying characteristics as those isolated through radiation selection by Welch and Maxcy (20).

Sweet and Moseley (17) have presented strong evidence indicating that the recombination repair system in *M. radiodurans* is accurate, in contrast to the error-prone system in *Escherichia coli* B/r (9, 22). The results of the present study, although not indicating specific mutation frequencies, tend to indicate that highly radiation-resistant M-A also possess an accurate repair system. Therefore, the concerns expressed by Shapiro et al. (16) and Ito (8) that the pink micrococci, such as *M. radiodurans* and *M. radiophilus*, may have been altered by radiation selection appear to be unwarranted. However, when considering bacteria of intermediate radiation resistance, it is possible that radiation-induced mutations may be significant. Determinations of the mutation susceptibility of various isolates showing a wide range of radiation resistance should help to clarify the need for an alternative selective procedure.

The highly toxic nature of irradiated PCA on radiation-resistant bacteria was unexpected. Further work on this phenomenon and its relation to food processing is in progress.

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