Comparative Resistance of Nonsporogenic Bacteria to Low-Temperature Gamma Irradiation

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A total of 36 microorganisms, comprising 19 species of 11 genera, were screened for radiation resistance with $^{60}$Co gamma rays at a radiation temperature of $-80 \pm 2 \degree C$ in phosphate buffer (pH 7.0) under vacuum. *Micrococcus radiodurans* was the most resistant organism. An initial population of $2.8 \times 10^4$ cells per dose of this species survived 2.4 but not 2.7 Mrad. Of the remaining 18 species with initial populations of about $10^4$ cells per dose, *Streptococcus faecium* survived 0.9 to 1.5 Mrad, depending on the strain tested. *S. faecalis* QM survived 0.9 but not 1.2 Mrad. *S. faecalis* 1539 and *Alcaligenes faecalis* survived 0.6 but not 0.9 Mrad. Three species of *Salmonella*, one strain each of *Escherichia coli*, *Streptococcus lactis*, and *Aerobacter aerogenes* survived 0.3 but not 0.6 Mrad. The remaining 22 bacteria did not survive 0.3 Mrad, the lowest dose tested. Detailed survival curve determinations for four strains of *S. faecium*, the most resistant of these bacteria of public health significance, indicated the following order of resistance at $-80 \degree C$: $a_{21} > \theta_{12} = F_a > F_{ae}$. Each strain produced two exponential survival curves with different slopes, the breaks occurring at 0.3 to 0.5 Mrad. The D values (doses which reduce the microbial population by 90%) of the more resistant cell fractions were two- to three-fold higher than the more sensitive cell fraction. The resistance of strain $a_{21}$ was determined at different radiation temperature (+5, −30, −80, −140, −196 C). The D value-radiation temperature relationship followed a quadratic equation. Computations of $E_a$ and $Q_{10}$ values (activation energy and temperature coefficient, respectively) showed a very small thermodynamic effect on radiation death. An Arrhenius evaluation of the temperature effect on cell kill indicated that there was no simple physicochemical mechanism which might explain the change in D value as a function of temperature.

The reviews by Goldblith (15) and Grez et al. (16) provide considerable evidence that the radiation resistance of microorganisms increases as the temperature of irradiation decreases. In fact, the resistance of strain 33A *Clostridium botulinum* spores rises linearly in beef as the radiation temperature is lowered (17). The protective effect of decreasing temperatures may be explained by the progressive reduction in the migration to cell-sensitive sites of free radicals and excited molecules formed by the reaction of ionizing energy with the medium surrounding the cells (indirect lethal action). These entrapped chemical entities probably recombine and become nonlethal before the medium thaws.

The predominating microflora in raw and low-dose-irradiated food products are vegetative cells. These organisms are much more sensitive to radiation than sporeformers (5, 12, 19, 23, 27–30, 32), with the exception of *Micrococcus radiodurans* (2, 5). However, it is conceivable that progressively decreasing radiation temperatures may produce a more rapid rate of increased resistance among vegetative bacteria than among spores, so that at some cryogenic temperature certain food spoilage nonsporeformers may equal, if not surpass, the radiation resistance of some spores. For example, Coleby et al. (8) apparently found streptococci in one swolgen can of pork out of 140 cans irradiated to 5 Mrad at $-75 \degree C$, whereas swolgen cans which had received 3 Mrad at room temperature contained clostridial spores. They cautioned that, at cryo-
genic temperatures, vegetative organisms such as fecal streptococci may be more of a radiation problem than sporeforming bacteria. Hence it was of interest to determine the influence of successive lowering of radiation temperatures on representative vegetative bacteria found in foods.

This communication reports on (i) the comparative radiation resistance of some 19 species of vegetative bacteria, (ii) the effect of radiation temperature on the sensitivity of *Streptococcus faecium*, and (iii) the behavior of the survival curve of this organism at various radiation temperatures.

**MATERIALS AND METHODS**

**Test organisms.** A total of 36 nonsporogenic microorganisms, comprising 19 species of 11 genera, were selected from our collection. They included 20 gram-negative and 16 gram-positive test organisms (Tables 1 and 2). Most of them had been isolated from various food products. The stock cultures were kept in Trypticase soy agar (TSA) slants at 2 to 5 C.

*M. radiodurans* was initially obtained through the courtesy of A. W. Anderson, Oregon State University, Corvallis, Ore., and *S. faecium* strains 221, 121, and 12 were secured from F. R. Kronenwett, American Biological Control Laboratories, Inc., Tenafly, N.J., who obtained them from E. A. Christensen, Statens Seruminstitut, Copenhagen.

**Culture preparation.** Each test organism was inoculated by needle from the stock slants into 12 ml of Trypticase soy broth (TSB). After incubation for 24 h at 30 C, 5 ml of the culture was added to each of two 1-liter flasks containing 500-ml quantities of TSB. The two flasks were incubated for 24 h at 30 C, the cultures were centrifuged at 2,520×g for 20 min at 2 to 5 C, and the pellet was washed twice by resuspension and centrifugation in 0.067 M Sorensen phosphate buffer (pH 7.0). The cells were stored in 60 to 80 ml of the buffer at 2 to 5 C. Under these conditions no changes in radiation resistance were detected with any of the organisms for the duration of the study.

**Enumeration of cell suspensions.** The cell populations were determined in duplicate by preparing decimal dilutions of the cell suspensions in 0.1% peptone (Difco) and plating triplicate dilutions on TSA. The plates were incubated for 48 h at 30 C, and the six replicate plate counts (30 to 300 colonies) were averaged.

**Radiation resistance.** To screen the bacteria for comparative radiation resistance, each stock suspension was diluted in chilled buffer to about 10⁴ cells/ml, dispensed aseptically in 1.0-ml quantities into sterile cotton-plugged Pyrex tubes (10 by 75 mm) and then frozen in a dry ice-acetone bath (−80 C). Ten tubes were inserted into a polystyrene holder (which is essentially unit density) held in a no. 2½ (401 × 411 inches) metal can and vacuum sealed to about 150 mm of Hg pressure (25-inch vacuum). The holder contained two parallel rows of five holes, so that two organisms (five replicate tubes per organism) were placed in each can. In addition, to study the effect of radiation temperature on four strains of *S. faecium*, 5.5 ml of test suspension was aseptically dispensed into sterile Pyrex tubes (16 by 150 mm), sealed under vacuum (150 mm of Hg pressure) in a tube sealer (O. P. Snyder, Jr., Ph.D. thesis, Univ. of

**Table 1. Resistance to gamma rays** of some nonsporogenic bacteria

| Organism                    | Strain     | Cells per tube (×10⁴) | No. of tubes (out of 5) showing growth at Mrad doses: |
|-----------------------------|------------|----------------------|------------------------------------------------------|
|                             |            |                      | 0  | 0.3 | 0.6 | 0.9–1.8³ |
| *Pseudomonas aeruginosa*    | QMB1468    | 3.2                  | 5  | 0   | 0   | 0         |
| *Serratia marcescens*       | QMB1466    | 1.9                  | 5  | 0   | 0   | 0         |
|                             | QMB1465    | 1.8                  | 5  | 0   | 0   | 0         |
| *Proteus vulgaris*          | QMB1563    | 0.4                  | 5  | 0   | 0   | 0         |
| *Aerobacter aerogenes*      | type I     | 0.9                  | 5  | 0   | 0   | 0         |
|                             | 4211-65    | 2.5                  | 5  | 0   | 0   | 0         |
|                             | 3228-65    | 2.7                  | 5  | 1   | 0   | 0         |
| *Escherichia coli*          | B          | 2.0                  | 5  | 0   | 0   | 0         |
|                             | B/r        | 1.9                  | 5  | 0   | 0   | 0         |
|                             | 198        | 2.2                  | 5  | 0   | 0   | 0         |
|                             | 2985-66    | 2.0                  | 5  | 0   | 0   | 0         |
|                             | 1711-64    | 1.9                  | 5  | 0   | 0   | 0         |
|                             | 3543-66    | 2.1                  | 5  | 5   | 0   | 0         |
| *Salmonella typhimurium*    | RIA        | 2.1                  | 5  | 0   | 0   | 0         |
| *Salmonella infantis*       | 165        | 1.5                  | 5  | 0   | 0   | 0         |
| *Salmonella panama*         |            | 2.5                  | 5  | 0   | 0   | 0         |
| *Salmonella javiana*        |            | 2.3                  | 5  | 4   | 0   | 0         |
| *Salmonella heidelberg*     |            | 1.7                  | 5  | 1   | 0   | 0         |
| *Salmonella senftenberg*    |            | 2.1                  | 5  | 0   | 0   | 0         |
| *Alcaligenes faecalis*      | QMB1483    | 3.2                  | 5  | 5   | 4   | 0         |

³ Radiation temperature −80 ± 2 C.

³ Doses increase in 0.3 Mrad units.
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TABLE 2. Resistance to gamma rays* of some gram-positive nonsporogenic bacteria

| Organism                  | Strain | Cells per tube (x 10^3) | No. of tubes (out of 5) showing growth at Mrad doses: |
|---------------------------|--------|-------------------------|-------------------------------------------------------|
|                           |        |                         | 0   | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 | 1.8 |
| Staphylococcus aureus     | 1458   | 2.0                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
|                           | 293    | 1.5                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
|                           | 237    | 1.2                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
|                           | 315    | 1.1                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
|                           | 478    | 0.9                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
|                           | 493    | 0.7                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
| Lactobacillus casei       | 1475   | 1.0                      | 5   | 0   | 0   | 0   | 0   | 0   | 0   |
| Lactobacillus arabinosus  | 1467   | 0.9                      | 5   | 4   | 0   | 0   | 0   | 0   | 0   |
| Streptococcus lactis      | 1539   | 1.0                      | 5   | 5   | 1   | 0   | 0   | 0   | 0   |
| Streptococcus faecalis    | FEC    | 2.0                      | 5   | 5   | 5   | 5   | 0   | 0   | 0   |
|                           | E12    | 2.5                      | 5   | 5   | 5   | 5   | 0   | 0   | 0   |
|                           | α21    | 2.1                      | 5   | 5   | 5   | 5   | 5   | 3   | 0   |
|                           | Fα     | 2.8                      | 5   | 5   | 5   | 5   | 2   | 1   | 0   |
| Micrococcus radiodurans   | FEC    | 0.3                      | 5   | 5   | 5   | 5   | 5   | 5   | 5   |

* Radiation temperature -80 ± 2°C.

Massachusetts, Amherst, 1969) and frozen in dry ice-acetone.

Each can of 10- by 75-mm tubes was irradiated with 60Co gamma rays at -80 ± 2°C in the range 0 to 1.8 Mrad in increments of 0.3 Mrad. Duplicatesealed 16- by 150-mm tubes were irradiated from 0 to 0.2 Mrad in dose intervals of 0.05 Mrad and thereafter in 0.1 Mrad units up to 1.2 Mrad. All doses varied only by ±2%. The latter samples were irradiated at 5, -30, -80, -140, and -196°C which were controlled (to ± 2°C) and monitored as described by Jarrett (21).

Recovery of irradiated cells. After irradiation, all samples were refrigerated overnight at 2 to 5°C. One-milliliter quantities of double strength TSB were then added aseptically to each of the cotton-plugged tubes, incubated for 14 days at 30°C, and observed for turbidity. Longer periods of incubation resulted in excessive evaporation of the tube contents even when the incubator was humidified. Where necessary for confirmation, tubes containing Streptococcus species were subcultured in azide dextrose broth for 24 h, then in ethyl violet azide broth for 24 h, followed by simultaneous subculturing into 6.5% NaCl tryptose broth and tellurite tryptose broth for 48 h. Incubation was at 30°C. Microscope examinations for the presence of gram-positive cells were made from the NaCl tryptose tubes. No additional confirmatory tests were performed since the organisms were initially pure cultures.

The vacuum-sealed large tubes were used to determine the survival of cells as a function of dose and radiation temperature. The irradiated tube contents were thoroughly mixed on a Vortex mixer and opened aseptically. Duplicate 1.0-ml samples per tube were enumerated for viable cells as above. Samples exposed to higher doses were also plated with undiluted samples in triplicate. The average colony counts of the six replicate plates per dilution were plotted as log survivors versus dosage.

Data processing. The D value, or the dose which reduces the microbial population by 90% (one log cycle on semilog coordinates), was obtained from the survival curves by inspection. The thermodynamic functions of the influence of radiation temperature on spore death, activation energy (Ea), and temperature coefficient (Q10) were computed as cited previously (17).

RESULTS

Range of resistance of 19 species to gamma irradiation. Table 1 indicates the comparative radiation resistances of the 20 gram-negative organisms. Alcaligenes faecalis QMB 1483 was the most resistant, surviving 0.6 but not 0.9 Mrad. Escherichia coli 3543-66, Salmonella javiana, S. heidelberg, and Aerobacter aerogenes 3238-65 were less resistant, in that order, than A. faecalis QMB 1483; they survived, to varying degrees, 0.3 but not 0.6 Mrad. The remainder of the organisms were inactivated by 0.3 Mrad.

Among the gram-positive group (Table 2), M. radiodurans, as expected, was the most radiation resistant. It survived 1.8 Mrad, the highest dose employed, even though the initial cell population was only 3 x 10^4 per tube. S. faecium was the next most resistant species tested, surviving at least 0.9 Mrad up to 1.5 Mrad, depending on the strain used. Streptococcus faecalis resisted 0.6 to 0.9 Mrad, while S. faecium survived 0.3 but not 0.6 Mrad, whereas Staphylococcus aureus, Lactobacillus casei, and Lactobacillus arabinosus did not survive even 0.3 Mrad.

Comparative resistance of S. faecium strains. To the best of our knowledge, M.
**radiodurans** is neither a food spoilage organism, a public health hazard, nor a measure of food sanitation. Moreover, it is heat sensitive (D_{10} = 0.75, z = 10.65); the calculations by Duggan et al. (10) indicate that a thermal process of 65.6 C (150 F) for 2 min should reduce the contamination cell level in raw beef by a factor of 10^{-28}. Since meat prepared for radiation sterilization is heated to a center temperature of 70 C (158 F) to inactivate the meat enzymes, _M. radiodurans_ would not be expected to present a radiation problem. Hence no additional radiation studies were performed with this organism. On the other hand, _S. faecium_ is a member of the Lancefield group D streptococci which are normally present in mammalian feces and is frequently found in foods. (Some investigators have suggested that it be used as an indicator of microbiological quality of foods and food plant sanitation [31].) In addition, its heat resistance is apparently relatively high (3, 9, 18, 20, 22, Drake, S. D., J. B. Evans, and C. F. Niven, Jr., Bacteriol. Proc., p. 24, 1958). Therefore the four strains of _S. faecium_ were selected for additional studies.

Comparative survival data of the four strains of _S. faecium_ were obtained at a radiation temperature of -80 ± 2 C. The results were plotted on linear, semilog, and log-log coordinates. Linear regression analysis indicated that a curve with two different exponential slopes (A and B) gave the best fit to the data of each organism, the break in the slopes occurring at 0.3 to 0.5 Mrad, depending on the strain tested. A typical plot (Fig. 1) shows that strain a21 has a D value of both 0.095 (slope A) and 0.315 (slope B), the break in the curve occurring at 0.3 Mrad.

Table 3 presents the D values and linear regression equations (log y = -mx + b, where m is the slope and b is the intercept on the y axis) of the survival curves of the four strains. The D values of the first portion of the curves (slopes A) were approximately similar (strain FEC was somewhat more sensitive than the other strains), whereas the second part of the curves (slopes B) were shallower but differed among the strains. The apparent order of resistances, based on slopes B, were strain a21 > ø12 > Fø > FEC, with D values of 0.32, 0.22, 0.19, and 0.13, respectively.

**Effect of radiation temperature on the resistance of _S. faecium_.** Strain a21 was used to determine the effect of varying the temperature of irradiation on its resistance. All five temperatures employed (5, -30, -80, -140, -196 C) produced curves with two exponential survival slopes (A and B). The D values of the B slopes were two- to threefold higher than the D values of the A slopes (Table 4). The change in resistance with radiation temperature is illustrated in Fig. 2 for both parts of the curves. The more resistant B slope was considerably more dependent on radiation temperature than the initial A slope.

A computation of the _E_ and _Q_ values based upon the B slopes indicated a relatively small thermodynamic effect on the radiation

![Graph](http://aem.asm.org/)

**Fig. 1. Resistance of Streptococcus faecium a21 to gamma rays at a radiation temperature of -80 ± 2 C.** This curve represents 5.5 ml of cell suspension per tube, 2 tubes per dose, 2 plate counts per tube, average of 3 plates per dilution.

**Table 3. Comparative radiation resistance of some Streptococcus faecium strains**

| _S. faecium_ strain | Slope A | | Slope B |
|---------------------|---------|---------|
|                     | D value* (Mrad) | Regression equation | D value (Mrad) | Regression equation |
|                     | m | b    |         | m | b    |
| Fø                  | 0.12 | -8.37 | 7.36   | 0.19 | -5.28 | 6.28   |
| a21                 | 0.10 | -10.31 | 6.89   | 0.32 | -3.13 | 4.73   |
| ø12                 | 0.11 | -9.52 | 7.43   | 0.22 | -4.62 | 4.77   |
| FEC                 | 0.08 | -13.84 | 6.99   | 0.13 | -8.10 | 5.19   |

* Radiation temperature -80 ± 2 C.
* Obtained from the survival curve by inspection, not by computation of 1/m.
* Log y = -mx + b.
TABLE 4. Effect of radiation temperature on the resistance of Streptococcus faecium a21 to gamma rays

| Radiation temp (°C) | Slope A | Slope B |
|---------------------|---------|---------|
|                     | D value* (Mrad) | Regression equation* | D value* (Mrad) | Regression equation* |
|                     | m       | b       | m     | b       |
| 5                   | 0.03    | -30.38  | 7.35  | 0.09    | -10.89  | 5.59   |
| -30                 | 0.11    | -9.12   | 7.33  | 0.24    | -4.17   | 5.78   |
| -80                 | 0.10    | -10.13  | 7.39  | 0.33    | -3.09   | 4.90   |
| -140                | 0.14    | -7.36   | 7.07  | 0.37    | -2.72   | 5.20   |
| -196                | 0.15    | -6.58   | 7.45  | 0.38    | -2.64   | 5.47   |

* Obtained from the survival curve by inspection, not by computation of 1/m.
* Log y = -mx + b.

**DISCUSSION**

Even at a radiation temperature of -80°C, where inactivation of bacteria by indirect action is significantly reduced, important food contaminants such as *S. aureus*, *E. coli*, *Salmonella*, *Pseudomonas*, or *Lactobacillus* remain very radiosensitive. As reported by other investigators (12, 23, 24, 32), the most radioresistant vegetative organisms were members of the gram-positive group, although there appeared to be considerable overlapping in resistance with the gram-negative group (Tables 1 and 2).

**TABLE 5. Effect of radiation temperature on death kinetics of Streptococcus faecium a21**

| Radiation temperature (°C) | D value* (Mrad) | Ea (cal/mol) | Q10 |
|---------------------------|------------------|--------------|-----|
| -196                      | 0.38             | 10           | 1.01|
| -140                      | 0.37             | 97           | 1.02|
| -80                       | 0.33             | 594          | 1.07|
| -30                       | 0.24             | 3765         | 1.32|
| 5                         | 0.09             |              |     |

* The more resistant, or B portion, of the survival curve was used for analysis.
* Obtained from the survival curve by inspection.

**FIG. 3. Arrhenius relationship between radiation resistance and radiation temperature of Streptococcus faecium a21.**
Among the microorganisms of public health significance, tested, the four *S. faecium* strains were the most radiation resistant. This agreed with the observations of Christensen (4), Matsuyama et al. (26), and Niven (27).

Three general forms of survival curves are encountered when different organisms are inactivated by radiation under varying conditions, namely, type A which is exponential, type B which has a resistant "tail" (concave), and type C which is convex, on a semilogarithmic plot (1). The latter may be either subtype C1 (a shoulder followed by an exponential portion) or subtype C2 (without the exponential portion).

Christensen and co-workers (4, 6, 7), using strain 012 and F4 and other strains of *S. faecium* dried in serum broth or in buffered saline and irradiated in air, obtained type C2 survival curves. Matsuyama et al. (26) observed type C1 survival with their *S. faecium* strains suspended in heart infusion broth and irradiated either aerobically or anaerobically, at ambient temperature or at -79 C. Ley et al. (25) also obtained the C1 form with *S. faecium* cells suspended in water or in buffer and irradiated in air at ambient temperature.

Although the double exponential (type B) form of survival curve for *S. faecium* had been encountered with a heat process (20), this kind of radiation dose response for this organism (Fig. 1) apparently has not been described heretofore. The vast majority (99.9%) of the cells were relatively radiosensitive, whereas the last 0.1% (some 5,000 to 10,000 cells in these experiments) produced a "tail" two- to threefold more resistant than the total population (Table 3). That the data are valid is indicated by the correlation coefficient (practically 1.0), standard error of estimate (0.1), and confidence intervals at the 95% level (0.2) tabulated in Fig. 1. This kind of low variability of experimental information generated was also reflected at all other radiation temperatures tested. That the dose response curves of *S. faecium* strains varied with the investigator is not surprising, since radiation damage and cell recovery can be modified by manipulating the environment of the organism at the various stages of the handling procedure (1).

Our type B curves may be interpreted in several ways. (i) The tail cells are resistant mutants, although Erdman et al. (13) required repeated cycling of irradiation and subculturing of survivors to demonstrate increased resistance over the original cultures. Our studies were conducted on the initial populations only. (ii) A cell culture in the logarithmic growth phase is usually more sensitive than one in the stationary phase, so that a nonsynchronous-growing population containing both kinds of cells will be heterogeneous with respect to their radioresistances. (iii) Dissolved oxygen in a solution enhances microbial radioresitivity; even the small quantity of oxygen present in "tank" nitrogen can affect cell resistance (1). Our method of preparing samples for irradiation certainly did not render our sealed suspensions completely anoxic. Low-dose (about 0.1 Mrad) irradiation at room temperature depletes all oxygen even from air-saturated solutions (14); hence the initial steep slope of our *S. faecium* survival curves followed by a shallower slope (or more resistant "tail") after the residual oxygen in the sealed tubes was depleted and the samples became anoxic. Or (iv), in accordance with target theory, the bacterial population may have dissimilar cells of two different radiosensitive targets. Figure 1 may indicate how the survival curve is decomposed into two exponentials which reflect the two target sizes in these populations.

The above interpretations for the occurrence of our type B curves are all amenable to verification by additional experiments, but it was not in our interest to pursue such studies.

The thermodynamic effect (Ea and Q10) on radiation inactivation of *S. faecium* α21 was very small in the range -196 to -30 C (Table 5). This agrees with previous observations for spores of *C. botulinum* suspended in beef and irradiated at cryogenic temperatures (11, 17). The transition from ice (-30 C) to the liquid state (5 C) produced higher Ea and Q10 values, which might be associated with the diffusion of small free radicals in this temperature range.

An Arrhenius plot of the death rate data as a function of radiation temperature produced a smooth unbroken curve (Fig. 3) comparable to that obtained for our beef-inoculated pack (17). Whether our model is a simple phosphate buffer system using *S. faecium* vegetative cells or a complex food system containing *C. botulinum* spores, the physicochemical mechanism taking place which might account for the change in bacterial death with temperature is apparently not a simple phenomenon.

A linear plot of the D values vs. radiation temperatures of both the A and B portions of the survival curves of strain α21 produced two convex curves (Fig. 2). The change in radiosensitivity of the more resistant tail (Fig. 2, curve B) portion of the curves was much more dependent on the change in radiation temperature than the more radiation-sensitive initial
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(2) portion. A statistical analysis of these two curves showed that the best relationship between D values and temperatures was a quadratic with the general equation y = –ax^2 – bx + c, where a, b, and c are constants (Fig. 2).

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