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Lewis Keller
University of Nebraska-Lincoln

R. Burt Maxcy
University of Nebraska-Lincoln

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Effect of Physiological Age on Radiation Resistance of Some Bacteria That Are Highly Radiation Resistant†

LEWIS C. KELLER‡ AND R. BURT MAXCY*

Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska 68583-0919

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Physiological age-dependent variation in radiation resistance was studied for three bacteria that are highly radiation resistant: *Micrococcus radiodurans*, *Micrococcus* sp. isolate C-3, and *Moraxella* sp. isolate 4. Stationary-phase cultures of *M. radiodurans* and isolate C-3 were much more resistant to gamma radiation than were log-phase cultures. This pattern of relative resistance was reversed for isolate 4. Resistance of isolate 4 to UV light was also greater during log phase, although heat resistance and NaCl tolerance after heat stress were greater during stationary phase. Radiation-induced injury of isolate 4 compared with injury of *Escherichia coli* B suggested that the injury process, as well as the lethal process, was affected by growth phase. The hypothesis that growth rate affects radiation resistance was tested, and results were interpreted in light of the probable confounding effect of methods used to alter growth rates of bacteria. These results indicate that dose-response experiments should be designed to measure survival during the most resistant growth phase of the organism under study. This timing is particularly important when extrapolations of survival results might be made to potential irradiation processes for foods.

Large variations in radiation survival exist among bacterial strains as reported by different authors. These variations appear to be mostly independent of the physicochemical conditions existing during irradiation, suggesting that physiological factors may be the cause. Differences in radiation resistance related to the growth phase of cultures have been observed for *Micrococcus radiodurans* (10; D. E. Duggan, Ph.D thesis, Oregon State University, Corvallis, 1961), *Escherichia coli* (1, 9, 11, 12, 15), and *Salmonella typhimurium* (2). Although these reports and others have demonstrated that the physiological state of pure cultures affects radiation resistance, this important factor has not been taken into account in the design of experiments to determine relative radiation resistance of different bacteria. It is likely that this lack of proper methodology has resulted in flawed comparisons among survival data for different bacteria. As an example, relative radiation resistance data compiled by Ingram and Farkas (4) do not include knowledge of growth phases at the time of irradiation for the cultures studied.

This paper presents data showing the importance of growth-phase effects on dose-response relationships, and it shows that different bacteria can have different patterns of survival response with respect to growth phase. Also discussed is the hypothesis that radiation resistance of an organism is affected by growth rate.

MATERIALS AND METHODS

Organisms and culture. Four radiation-resistant bacteria were used: *M. radiodurans* (ATCC 13939), a *Moraxella-Acinetobacter* sp., isolate 4 (16), and a *Micrococcus* sp., isolate C-3 (17). Isolate 4 has characteristics of the M-5 group of unclassified *Moraxella* spp. from clinical specimens described by Tatum et al. (13). A nonresistant bacterium, *E. coli* B, was also used in some comparative experiments.

Plate count agar (PCA; Difco Laboratories) was used for propagation of each organism, and subcultures were inoculated into m-Plate Count Broth (Difco). Incubation was at 32°C, and since the isolates used were aerobic, shaking was employed to enhance growth.

Growth rate determination. Growth rates of cultures were determined by both viable counts with PCA and with turbidity measurements. The turbidity of isolate C-3 and isolate 4 cultures was measured at a wavelength of 560 nm. The cultures were diluted in 0.2 mM phosphate buffer (pH 7.2) to give an absorbance of less than 0.04. The absorbance readings were then multiplied by the dilution factor to calculate the true absorbance (7).

Maximum growth rates for the exponential phase of growth of each culture were estimated by the method of least squares, and the coefficients were used to calculate cell doubling times.

Irradiation. A ^{60}Co irradiator of the design described by Teeny and Miyauchi (14) was used for gamma irradiation. Cultures in test tubes (10 by 75 mm) were either irradiated at ambient air temperature or quick-frozen in dry ice-acetone and kept at ca. −30°C during irradiation by utilizing dry ice in the sample carrier. During any delay before or after irradiation, the frozen cultures were held at −25°C. Frozen cultures for plating or further treatments after irradiation were thawed by rolling the culture tubes between the hands of the experimenter. Dilutions for plate counts were then made in dilution buffer (0.2 mM phosphate buffer, pH 7.2). PCA plates were incubated for 5 days at 32°C before counting to allow recovery and growth of irradiated cells (8). Surviving fractions were calculated by dividing irradiated counts by the corresponding unirradiated control counts.

UV irradiation of isolate 4 was done by suspending cells in 0.2 mM phosphate buffer (pH 7.2) at room temperature. The incident dose rate was measured with a YSI-Kettering radiometer (model 65A) at 22 J m^{-2} s^{-1} (33 cm from a General Electric G15T8 germicidal lamp). All suspensions were agitated by a magnetic stirrer during irradiation.

Heating. Cultures to be tested for heat resistance were added to test tubes (10 by 75 mm) which were then partially immersed in a 70°C water bath for specified time intervals.

* Corresponding author.
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‡ Present address: Frito-Lay, Inc., Irving, TX 75061.

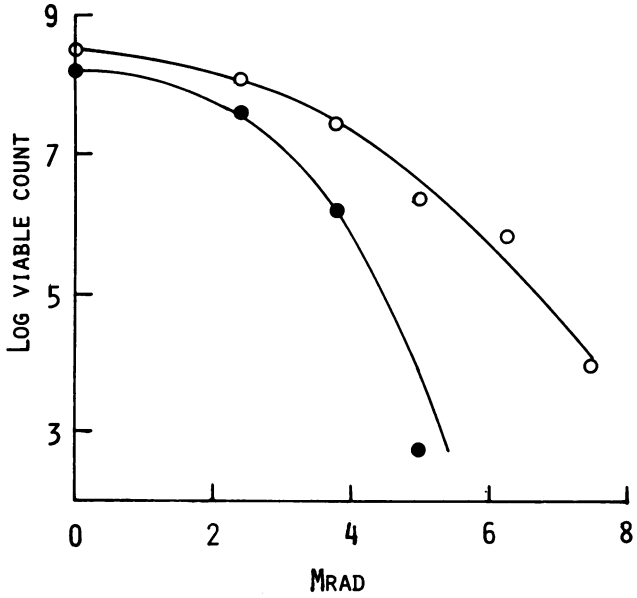

FIG. 1. Irradiation survival of isolate C-3 cultures harvested during apparent maximum cell density at different incubation times: 26 h (○) and 36 h (●). Actual data points are shown, with curves plotted by the method of King et al. (6).

RESULTS

Radiation survival of C-3 cultures. Gamma irradiation of isolate C-3 was done with cultures grown to approximately maximum cell density. A large disparity existed between the survival curves of different replicate samples harvested after different incubation times, but still at apparent maximum density (Fig. 1). The differences were much larger than could be attributed to experimental error. Under the propagation conditions used in these experiments, both the 26- and 36-h cultures were in approximately stationary phase of the growth cycle.

Survival of bacterial cultures. Physiological ages of cultures were determined from growth curves obtained by standardized culture propagation methods (Fig. 2). Based on the growth curve of each strain, sampling times were chosen to obtain cultures for irradiation during log and stationary phases. Cultures harvested after 16 h of incubation were used to represent mid-log phase. Early stationary- and late stationary-phase cultures were obtained at 36 and 48 h, respectively, for *M. radiodurans* and isolate C-3, and at 48 and 56 h for isolate 4. A 4.0-megarad radiation dose was used to cause significant killing of cultures in their most-resistant phase while ensuring that cultures in the least-resistant phase would have sufficient survivors for enumeration. Survival ratios of cultures irradiated with 4.0 megarads are given in Fig. 2. *M. radiodurans* and isolate C-3 were much more radiation resistant during early stationary phase than during log phase. Isolate 4, however, was more resistant during log phase than during stationary phase.

The enhanced log-phase resistance of isolate 4 was not expected in light of the current knowledge of growth phase-related survival characteristics of bacteria exposed to lethal treatments. Therefore, isolate 4 cultures were also studied for resistance to UV radiation and to heat and for NaCl tolerance after heat stress during the two growth phases (Fig. 3 and 4). Survival after UV irradiation was greater during log phase, but heat resistance and NaCl tolerance were greater during stationary phase. Isolate 4 cultures were far more sensitive to heat (70°C for 2 min) during log phase (log surviving fraction = −3.0) than during stationary phase (log surviving fraction = −0.2).

It was also of interest that isolate 4 was equally susceptible to radiation injury in either log phase or stationary phase. In contrast, *E. coli* B was 10 times more susceptible to injury during log phase than during stationary phase (Table 1). Furthermore, it appeared that the recovery rate of injured isolate 4 was the same for exponential- and stationary-phase cultures, unlike injured *E. coli* B, which had a higher recovery rate for log phase than for stationary phase (Fig. 5).

Effect of growth rate on radiation resistance. A hypothesis by Freedman and Bruce (3) proposes that growth rate affects radiation resistance of bacteria. In the present study, the

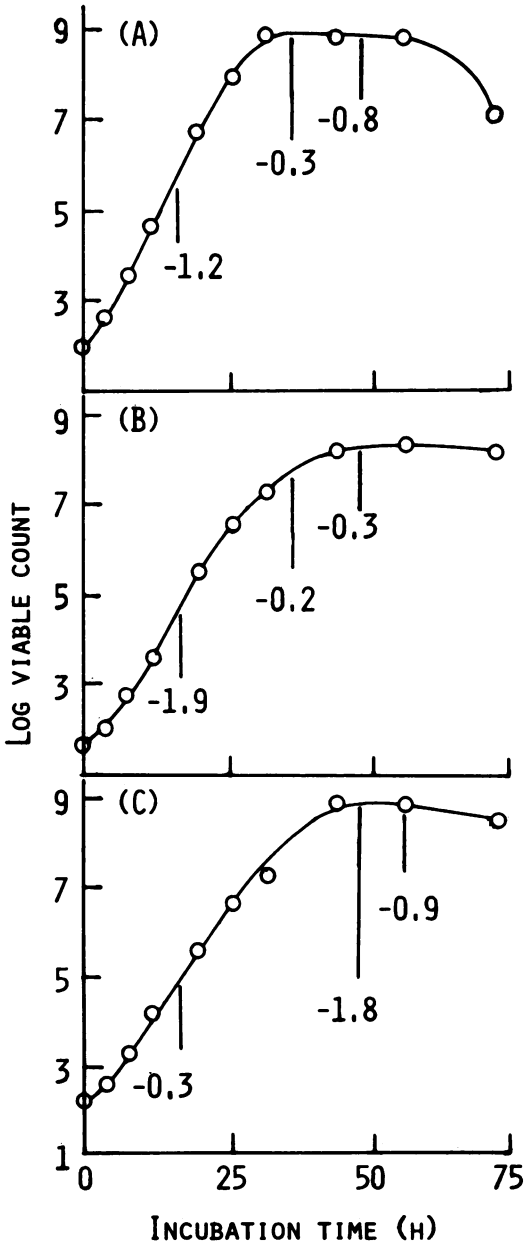

FIG. 2. Growth in m-Plate Count Broth at 32°C with shaking. (A) *M. radiodurans*, (B) isolate C-3, and (C) isolate 4. Log surviving fraction after 4.0 megarads of γ irradiation is shown for the sampling times indicated.

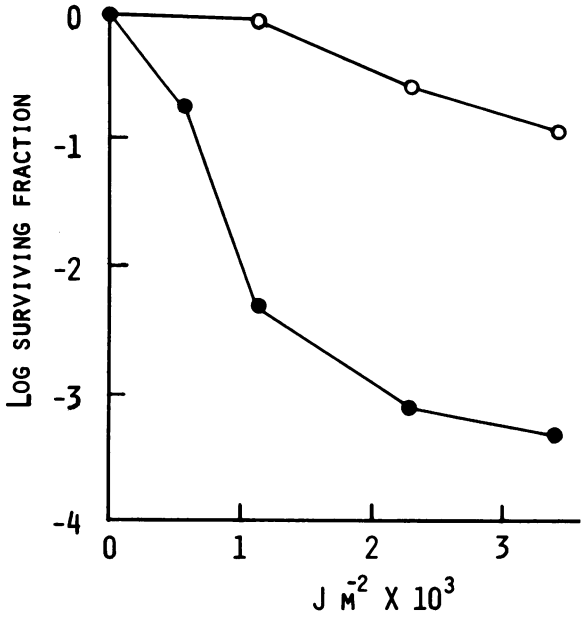

FIG. 3. UV survival of exponential-phase (○) and stationary-phase (●) cultures of isolate 4.

hypothesis implies that some factor or factors affecting growth rate could be responsible for the difference in radiation resistance during log phase and stationary phase. This hypothesis was tested for isolate C-3 by growing cultures at a reduced temperature to obtain slow growth independent of the growth-retarding or -promoting effects of differing growth media (3). The results are given in Fig. 6. Survival was reduced significantly in both exponential- and stationary-phase cultures grown at 18°C. However, large differences between survival rates of exponential- and stationary-phase cultures were still apparent for the slowly growing cultures of isolate C-3.

DISCUSSION

Implications of anomalous survival pattern of isolate 4. *Micrococcus* sp. isolate C-3 and *M. radiodurans* were more susceptible to irradiation killing during log-phase growth, as could be expected in light of the current knowledge of growth phase-related resistance to radiation or heat in other bacteria. Isolate 4, however, exhibited the opposite pattern of resistance. No explanation of this phenomenon is apparent from the data, except that it occurs for both gamma and UV resistance but not for heat resistance. No similar results were found in the literature.

The anomalous radiation resistance pattern of isolate 4 with respect to growth phase vividly illustrates that radiation resistance experiments must be designed to take growth phase into account. Pure cultures for dose-response experiments should always be studied to determine the relative resistance of different growth phases, and this information should be included as part of the published results. We believe that survival data for the most resistant phase of growth should always be used for comparisons among different organisms. This is particularly important if data might be used to infer survival characteristics of bacteria in irradiation processing of foods.

The possible effect of growth phase-related physiological factors on injury and recovery characteristics of bacteria was not directly addressed by this research. However, it can readily be seen that study of injury-recovery processes would be important not only for correctly interpreting survival data, but also for elucidating radiation resistance mechanisms. If repair characteristics relative to growth phase are different in different bacteria, as the data suggest (Fig. 5), injury studies, as well as survival studies, should be designed to take this effect into account.

Relation of growth rate to resistance of isolate C-3. The observed change in radiation resistance of slowly growing isolate C-3, along with the effect of different culture media on resistance of *M. radiodurans* (3), illustrates a difficulty in relating pure-culture survival data to radiation resistance of bacteria in food microenvironments. Also, compositions and temperatures of media may cause different effects. Isolate C-3 had lower resistance at a reduced growth rate, whereas Freedman and Bruce (3) found increased resistance in *M. radiodurans* grown in a medium which decreased the growth rate. The effect of different medium compositions or different temperatures may unavoidably confound studies seeking to understand the effect of growth rate-related factors on radiation resistance. At the present time, reliance upon pure-culture studies requires the assumption that relative survival levels of different bacteria are essentially proportional in either pure cultures or food microenvironments.

Method for comparing lethal rates of different radiation-resistant and nonresistant bacteria. The method of King et al. (6) is useful for comparing death rate data among bacteria that are highly radiation resistant and have extensive shoulders on their death rate curves. This method makes compari-

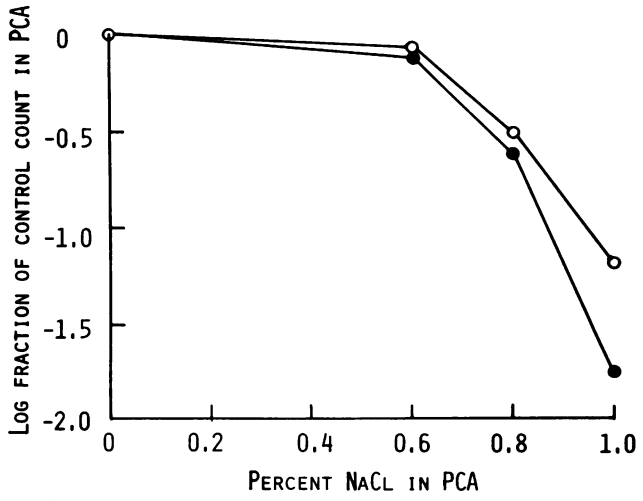

FIG. 4. Growth of isolate 4 inoculated onto PCA plates containing NaCl during exponential phase (●) and stationary phase (○) after heating at 70°C for 2 min.

TABLE 1. Extent of injury in γ-irradiated cultures of isolate 4 and *E. coli* B during different growth phases[a]

Organism	Growth phase (approx)	PCA count	PCAS[b] count	PCAS/PCA count (%)
Isolate 4	Exponential	5.1×10^3	1.9×10^2	3.7
	Stationary	7.6×10^7	3.4×10^6	4.5
E. coli B	Exponential	1.1×10^3	4.3×10^1	3.9
	Stationary	5.8×10^7	2.2×10^7	37.9

[a] Injury-inducing irradiation dose was 3.7 megarads for isolate 4 and 15 kilorads for *E. coli* B.

[b] PCAS is PCA + 0.85% NaCl for isolate 4 and PCA + 1.25% NaCl for *E. coli* B.

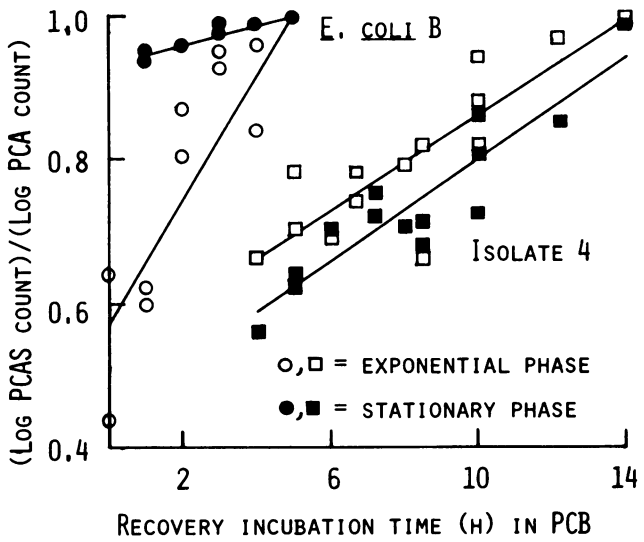

FIG. 5. Recovery kinetics after γ irradiation of isolate 4 and *E. coli* B incubated in m-Plate Count Broth before plating onto PCA and onto PCA with NaCl added (PCAS). Irradiation dose and NaCl concentration in PCA with NaCl was 3.7 megarads and 0.85% NaCl for isolate 4 and 15 kilorads and 1.25% NaCl for *E. coli* B.

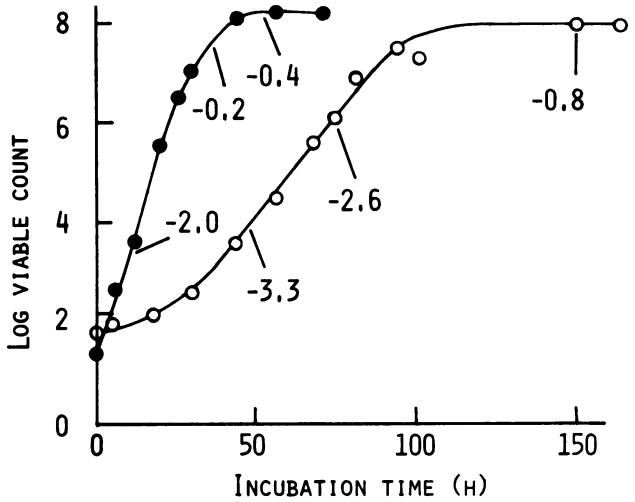

FIG. 6. Growth of C-3 in m-Plate Count Broth at 32°C (●) and 18°C (○). Log surviving fraction after 4.0 megarads of γ irradiation given for each sampling time indicated.

sons of resistance possible among organisms of widely varying resistance levels by using essentially the same concept as that used for heat processing for canned foods. With survival curves obtained under similar conditions of growth and irradiation, different organisms can be compared on the basis of calculated dose required to obtain a given log reduction in survival.

The UV survival curve of stationary-phase isolate 4 (Fig. 3) differs from γ irradiation survival curves. Rather than a shoulder followed by an accelerating death rate, the sigmoidal UV curve has a greatly diminished shoulder followed by a high death rate and then a shift to a lower death rate. This curve is suggestive of an inducible resistance system which becomes operative after irradiation is begun (5). The log-phase survival curve also exhibits this effect, although not as dramatically. These curves cannot be treated mathematically by the method of King et al. (6).

Laboratory studies of microorganisms in foods should be designed to account for possible growth phase-related effects on the attributes being studied. This applies for other treatments as well as those discussed in this paper, e.g., effects of chemical preservatives, sanitizers, simulated processing conditions, etc. Although methods to determine directly the growth phase of bacteria in nonaxenic food microenvironments are not obvious, knowledge of pure-culture response data in the highest and lowest phases of resistance might reveal useful correlations with food system observations. Pure-culture experiments could then be applied more accurately to predicting and understanding responses to irradiation and other factors affecting microorganisms in foods.

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