Increased Radiation Resistance of Vegetative *Bacillus pumilus*¹

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A 4.5-fold increase in vegetative cell radiation resistance of *Bacillus pumilus* E601, the internationally recognized biological standard for irradiation sterilization, was obtained by the repeated passage of resistant survivors through successive sublethal doses of ²⁶Co irradiation. This increase in resistance was accompanied by a corresponding increase in spore resistance through the seventh irradiation passage. By the fifteenth passage, the ability for spore formation was lost. Other effects noted by the successive irradiation dosages included loss of motility and pellicle formation, and changes in the Gram reaction, cell morphology, and colonial morphology. Increased resistance was also accompanied by an increased nutritional requirement for specific amino acids. Radiation resistance was not transferred from vegetative cells to spores.

Certification of efficacy of radiation sterilization processes has been based primarily upon the use of biological indicators, usually employing bacterial spores. The resistance of selected bacterial spores to irradiation has been characterized (13, 14). However, neither the resistance of corresponding biological indicator strain vegetative cells to repeated sublethal doses of irradiation, nor the effects of such sublethal irradiation on the cultural, morphological, and nutritional characteristics of vegetative cells, have been determined.

Prior to the isolation of *Micrococcus radiodurans* (1) and *Streptococcus faecium* (5), it was believed that bacterial spores were more resistant to radiation than were vegetative cells. Alterations in vegetative cell resistance produced by various irradiation procedures have not resulted in vegetative cells with resistance comparable to normal spores. Luckiesh and Knowles (12), in an early study of *Escherichia coli*, demonstrated a doubling of ultraviolet radiation resistance after five successive sublethal radiation treatments of surviving populations. They found no increase in resistance beyond the five passages. Gaden and Henley (9) found that E. coli 15 became more radiation sensitive after repeated gamma irradiation treatments whereas E. coli B and B/r increased in gamma radiation resistance under similar treatment. Erdman et al. (8) also showed development of resistance to gamma radiation in some strains of E. coli, *Streptococcus faecalis*, and *Clostridium botulinum* type A, but not in *C. botulinum* type E, *Staphylococcus aureus*, or *Salmonella gallinarum*. Even then, the maximal increase in radiation resistance never exceeded twofold. Recently it was reported that radiation-resistant mutants of *Salmonella typhimurium* show increased resistances to maxima of 12.5 and 20 times, respectively, for the D₁₀ (dose required to reduce the number of survivors one log number of the linear portions of the radiation survival curves) and 90% lethal dose values as compared to the original strain (7).

Spores of *Bacillus pumilus* E601 were reported by Pepper et al. (13) and Fogarty and Borick (2) to be among the most radiation resistant of the numerous strains of aerobic and anaerobic sporeformers studied. The radiation resistance of spores of this *B. pumilus* strain has been well characterized by many investigators and is now generally accepted as the international biological indicator standard for radiation sterilization processes. Vegetative cell resistance of this strain has not been studied previously; therefore, our primary objectives (presented in part at the 73rd Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 6 to 11 May 1973) were to determine the extent to which the radiation resistance of vegetative cells of *B. pumilus* E601 can be altered by sublethal gamma irradiation and to determine what cultural, morphological, physiological, and nutritional changes accompany the alteration of resistance.

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MATERIALS AND METHODS

Vegetative cell preparations. Vegetative cell cultures of B. pumilus E601 (ATCC 27142), E. coli B (ATCC 29212), E. coli B/r (ATCC 29227), and M. radiodurans (ATCC 13939) were prepared for irradiation by the following standardized procedure. A 0.1-ml volume of a 24-h Trypticase soy broth (BBL) culture was transferred to a 250-ml Erlenmeyer flask containing 125 ml of sterile Trypticase soy broth. The inoculated flasks were incubated on a rotary shaker at 37 C for 24 h until the culture well into the stationary phase. Mature spores of B. pumilus E601 were not detected under these growth conditions until about 96 h. Cells were harvested by centrifugation and suspended in sterile normal saline at approximately 10^6 cells per ml, and 2.0-ml volumes were pipetted into sterile, screw-cap vials (15 by 45 mm).

Duplicate ampoules were prepared for each irradiation treatment level for both spores and vegetative cells.

Spore preparations. Spores of B. pumilus E601 were obtained by inoculating the surface of Trypticase soy agar (BBL) plates (25 ml in 15- by 150-mm plastic petri dishes). The plates were incubated at 37 C until sporulation exceeded 95%, which normally occurred in less than 7 days. Spores were washed from the agar surface with sterile, deionized water, centrifuged, and resuspended in sterile, deionized water to a concentration of about 10^7/ml; 2.0-ml volumes were pipetted into sterile, screw-cap vials identical to those used for vegetative cell irradiations.

Irradiation. The source of gamma rays for irradiation was 60Co in a Gammacell 220 irradiator (Atomic Energy of Canada Limited) at the Central Research Laboratories of Johnson & Johnson, New Brunswick, N.J. The dose rates in the Gammacell during the 6-month duration of the study decreased by normal radioactive decay from 1.58 to 1.50 Mrads/h. Survival curves for both vegetative cells and spores were obtained by plotting the surviving fractions of the initial challenge populations against irradiation doses. The best fit of the data to the linear portions of the survival curves was by the least squares method (6).

Development of resistance. The method used to develop increased radiation resistance of vegetative B. pumilus E601 was modified from that of Erdman et al. (8). Cells grown and prepared for irradiation as above were irradiated at increasing dose levels. A 0.1-ml volume from each dose treatment level was transferred to fresh medium, and new cells were grown. The cells grown from the highest dose treatment vial were harvested and treated as above, after which the irradiation procedure was repeated.

Nutritional studies. The nutritional requirements of B. pumilus E601 strains at the various stages of radiation resistance were compared to the parent strain. The method employed was a composite of those of White (14) and Knight and Proom (11). All media were made from deionized water (18 Mohms of resistance) and glassware for stock solutions was acid-cleaned and thoroughly rinsed in deionized water. Sterile, disposable plastic pipettes and test tubes were universally employed in all nutritional studies.

Ammonium basal medium contained (per liter): Na2HPO4, 12H2O, 10.4 g; KH2PO4, 2.4 g; (NH4)2SO4, 2.0 g; MgSO4.7H2O, 50 mg; MnCl2·4H2O, 4 mg; FeSO4·7H2O, 2.8 mg; and glucose, 10.0 g. The pH was 7.2 after sterilization. The basal medium without the salts of Mg++, Mn++, and Fe++ or the carbon source was autoclaved for 10 min at 121 C. The salts, dissolved together at 50× concentration in 0.01 N H2SO4, were autoclaved separately, as was a 25% glucose solution. These latter solutions and the basal medium were mixed aseptically after autoclaving.

Nutritional requirements were determined by adding supplements to the above complete medium. Vitamins were (per milliliter): biotin, 1 ng; folate acid, 2 ng; pyridoxine-hydrochloride, 0.5 μg; and calcium pantothenate, 0.5 μg. Trace salts were (per liter): CaCl2, 1.0 mg; CoSO4·7H2O, 1.0 mg; ZnSO4·7H2O, 1.0 mg; CuSO4·5H2O, 0.1 mg; H3BO3, 0.1 mg; and Na2MoO4·2H2O, 0.1 mg; they were added after autoclaving as a 100× solution in 0.01 N H2SO4. Individual amino acid requirements were determined by supplementing the medium containing complete basal ammonium-glucose salts with mixtures of the various amino acids.

Stock solutions (50×) of the individual amino acids were used to make the assay medium containing amino acids. Each complete assay medium lacked only one single amino acid. The 50× stock solutions employed the following amino acids (per milliliter): dl-alanine, 1.5 mg; dl-aspartic acid, 3.0 mg; l-glutamic acid, 7.0 mg; l-proline, 3.0 mg; dl-methionine, 1.5 mg; l-arginine-hydrochloride, 3.5 mg; glycine, 1.5 mg; dl-serine, 0.75 mg; dl-phenylalanine, 1.5 mg; dl-tryptophan, 0.5 mg; l-tyrosine, 3.0 mg; l-histidine-hydrochloride, 2.0 mg; l-isoleucine, 2.0 mg; l-leucine, 2.0 mg; dl-threonine, 3.0 mg; dl-valine, 3.5 mg; l-cysteine, 2.5 mg; and l-lysine-hydrochloride, 2.5 mg.

All nutritional assays were performed by adding 5.0 ml of test substrate to screw-cap (sterile; 16 by 160 mm) plastic, disposable test tubes (Bioquest). Assays were performed in triplicate. The inoculum was prepared by the method of Campbell and Williams (3). Vegetative cells of B. pumilus E601 were grown as indicated above for radiation treatment. Cells were harvested by centrifugation, washed three times with sterile 18-Mohm deionized water, and adjusted to a concentration of 2 × 10^6 cells per ml. Each tube was inoculated with 0.1 ml of this cell suspension, mixed, and incubated at 37 C for 72 h. Absence of growth at 72 h in a particular test medium was interpreted as indicating a requirement for the metabolite excluded from that medium.

Characterization of strains. Parent and radiation-derived strains were compared morphologically and physiologically by the procedure of Gordon et al. (10).

RESULTS

Development of resistance. Figure 1 illustrates the development of resistance in B. pumilus E601 after 23 successive radiation treatments of survivors. No increase beyond that obtained on the 23rd radiation treatment
was noted after three additional treatments, at which point this part of the study was terminated. As resistance increased, the survival curves showed a marked extension of the shoulder, along with a decrease in the slope of the exponential portion of the curve.

For comparison of common vegetative cell resistance, *E. coli* B and the ultraviolet- and X-ray-resistant mutant B/r (15) and *Micrococcus radiodurans* were grown and irradiated in the same manner as the vegetative *B. pumilus* strains (Fig. 2). The D_{10} values of the various strains presented in Fig. 1 and 2 are given in Table 1. Strain V-23 (23rd irradiation treatment culture) was shown to exhibit a 4.5-fold increase in resistance when compared to the parent (V-0) strain.

The stability of the most resistant strain (V-23) was determined by transferring the isolate 15 times in Trypticase soy broth and then determining the dose survival curve. The resistance remained unchanged from that of the freshly derived V-23 strain (Fig. 3).

**Radiation-induced changes.** *B. pumilus* is most readily distinguished from other *Bacillus* species since *B. pumilus* does not reduce nitrate or hydrolyze starch although it possesses strong catalase activity. Throughout the development of resistance, strains were routinely checked for those physiological traits in addition to being observed microscopically and macroscopically.

Most physical changes were detected by the third radiation treatment. These were a loss of motility accompanied by the loss of normal pellicle formation, and a change in cellular arrangement from predominantly single cells (stationary phase) to short chains with some elongated cellular forms. The elongated cells were not of the filamentous type occasionally observed for the parent strain after marginally

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**Fig. 1. Development of radiation resistance in vegetative *Bacillus pumilus* E601 after 23 irradiation treatments of surviving cell populations. Symbols: ○, V-0 (parent strain); ○, V-1; △, V-7; △, V-12; ■, V-15; and ▲, V-23.**

**Fig. 2. Comparative radiation resistances of *Bacillus pumilus* E601/V-0 (○), *B. pumilus* E601/V-23 (■), *Escherichia coli* B (▲), *E. coli* B/r (△), and *Micrococcus radiodurans* (○).**

**Table 1. Vegetative cell radiation resistance of derived strains of *B. pumilus* E601**

| Passage no. | D_{10} value (Mrad) |
|-------------|----------------------|
| V-0         | 0.021                |
| V-1         | 0.026                |
| V-7         | 0.055                |
| V-12        | 0.068                |
| V-15        | 0.072                |
| V-23        | 0.095                |

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**Fig. 3. Stability of developed radiation resistance in vegetative *Bacillus pumilus* E601/V-23 before passage in TSB (○) and after 15 successive passages in TSB (○).**
lethal $^{60}$Co radiation treatments. A change in colonial morphology from rough to smooth was an early alteration. The resistant cells also became gram variable earlier in the growth cycle when compared to the parent strain.

The ability of the resistance-developing strains to sporulate on Trypticase soy agar appeared to be normal up to the seventh radiation treatment, but thereafter the ability to sporulate rapidly decreased. By the 13th radiation treatment, sporulation decreased to less than 1%, and by the 15th treatment only an occasional spore could be detected.

Characterization of strain V-23. A comparison of strains V-0 and V-23 is presented in Table 2, where it may be noted that the sole physiological change was the loss of the ability of V-23 to grow in 7% NaCl broth.

Transfer of resistance from vegetative cells to spores. The vegetative cell resistances of V-0 and V-7 and the spores produced by the two strains are compared in Fig. 4. Strains of greater resistance were not studied because of the decrease in the ability to sporulate, as mentioned above. It was noted that there was no significant transfer in radiation resistance from vegetative cells to their spores by resistant vegetative cells.

Nutritional studies. As resistance to radiation was increased by repeated radiation treatments, the nutritional requirements became more exacting (Table 3). The parent strain was found to require only the addition of biotin as a growth factor to the basal ammonium-glucose medium. This strain was, therefore, competent to synthesize all its necessary amino acids. This is in agreement with earlier published data for other B. pumilus strains (11). At the seventh radiation treatment, the addition of two amino acids was essential. After the 23rd treatment,

![Graph showing survival fraction vs. dose (Mrad)]

**FIG. 4.** Parent (V-0) and irradiation-derived strain (V-7) vegetative and spore resistance of Bacillus pumilus E601. Dose-survival plots are shown for vegetative cells of V-0 (●) and V-7 (■) or for the spores of V-0 (○) and V-7 (□).

| Table 2. Characterization of parent (V-0) and an irradiation-derived strain (V-23) of B. pumilus E601 |
|---------------------------------------------------------------|-----------------|------------------|
| Determination                                                | V-0             | V-23             |
| Shape                                                      | Rods            | Rods             |
| Size                                                       | 1.0 by 3.0 μm   | 1.0 by 3.0 to 8.0 μm |
| Arrangement                                                | Single, pairs   | Singles, pairs, short chains |
| Spores                                                     | Central, oval   | (Rare) central, oval |
| Motility                                                   | +               | -                |
| Anaerobic growth                                           | -               | -                |
| Maximum temperature                                        | 44°C            | 44°C             |
| Hydrolize starch                                           | -               | -                |
| Reduction of NO₃⁻                                          | -               | -                |
| Utilization of citrate                                     | +               | +                |
| Utilization of propionate                                  | -               | -                |
| Catalase                                                   | +               | +                |
| Produces acetylmethyl carbinol                             | +               | +                |
| Produces dihydroxy acetone                                 | +               | +                |
| Produces indole                                            | +               | +                |
| Decomposition of casein                                    | -               | -                |
| Growth in 5% NaCl                                         | +               | +                |
| Growth in 7% NaCl                                         | +               | +                |
| Growth at pH 5.7                                          | -               | +                |
| pH in glucose broth                                        | 4.7             | 4.8              |
| Fermentation of glucose                                    | Acid            | Acid             |
| Fermentation of trehalose                                  | Acid            | Acid             |
| Fermentation of mannitol                                   | Acid            | Acid             |
| Fermentation of arabinose                                  | Acid            | Acid (weak)      |
six amino acids were essential. Interestingly, even after the numerous repeated radiation treatments, the nutritional requirements for growth were relatively simple.

The effects of temperature on the nutritional growth requirements of the parent (V-0) and strain V-23 were compared (Table 4). No differences were noted between the requirements at 25 and 37 C, but at the upper temperature limit of growth the parent strain showed the need for 6 amino acids and V-23 was found to require 12 amino acids. Even at the upper temperature limit, nutritional requirements are relatively simple.

**DISCUSSION**

The most valuable biological indicator strain is one in which the normal population does not exhibit extreme resistance variability and thus gives reproducible and predictable sterilization process information. A 4.5-fold increase in the radiation resistance of vegetative cells of surviving *B. pumilus* E601 was obtained by repeated irradiation treatments. The irradiation dosage which the most resistant strain could withstand is greater than that previously reported for most other cultures. It is noteworthy, however, that although radiation resistance increased in vege-

| B. pumilus | Requirements |
|------------|--------------|
| NH₄⁺, glucose, salts | Vitamins | Amino acids |
| Parent (V-0) | + | Biotin | None |
| V-7 | + | Biotin | Ile, Val |
| V-14 | + | Biotin | Ile, Val, Glu |
| V-23 | + | Biotin | Ile, Val, Glu, Met, Thr, Cys |

*37 C.

**TABLE 3. Nutritional requirements* of parent and **Co irradiation-derived vegetative strains of B. pumilus E601**

| Temp (C) | Strain | NH₄⁺, glucose, salts | Vitamins | Amino acids |
|---------|-------|----------------------|----------|-------------|
| 25      | V-0   | +                    | Biotin   | None        |
|         | V-23  | +                    | Biotin   | Ile, Val, Glu, Met, Thr, Cys |
| 37      | V-0   | +                    | Biotin   | None        |
|         | V-23  | +                    | Biotin   | Ile, Val, Glu, Met, Thr, Cys |
| 44      | V-0   | +                    | Biotin   | Ile, Val, Glu, Asp, Phe, Try |
|         | V-23  | +                    | Biotin   | Ile, Val, Glu, Met, Thr, Cys, Asp, Ser, Phe, Tyr, Try, Lys |

The procedure employed in this study to isolate and select strains of increased radiation resistance also resulted in creating strains with nutritional requirements more exacting than the parent untreated strain. The irradiation treatments apparently created sufficient genetic damage so that enzymes critical in the synthesis of certain amino acids were either altered to such an extent that they were non-functional or were lost altogether. The greater the number of irradiation treatments, the greater the requirements for supplemental amino acids. These increased nutritional re-

**TABLE 4. Effect of growth temperature on the nutritional requirements of parent (V-0) and an irradiation-derived strain (V-23) of B. pumilus E601**
requirements were most pronounced at the highest growth temperature tested.

It is concluded from this study that successive reexposure of the biological indicator B. pumilus does not give rise to vegetative cells with sporelike resistance. Additional studies on the development of radiation resistance in B. pumilus E601 spores are in progress.

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