

Characterization of *Bacillus pumilus* E601 Spores After Single Sublethal Gamma Irradiation Treatments¹

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Eighteen survivor strains of *Bacillus pumilus* E601 have been isolated after single sublethal irradiation treatments with ⁶⁰Co. Primary isolation was based on the loss of motility and pellicle formation. However, with subsequent subcultivation, eight isolates reverted back to the standard of exhibiting motility and pellicle formation. Characteristics of the isolates include alterations in spore radiation resistance and in the amino acid requirements for spore germination and outgrowth. Other alterations in cultural and physiological characteristics were found. Three of the isolates were asporogenous.

Bacillus pumilus E601 has become recognized as one of the challenge microorganisms used for the certification of commercial radiation sterilization cycles. This same strain is also recommended in U.S.P. XVIII for use as the biological indicator of choice for the routine monitoring of the efficacy of production radiation sterilization processes. A microbial strain can only be valuable as a biological indicator if it can be reliably detected after an ineffective sterilization treatment. In related experimental studies, substerilization process dose treatments are often employed to produce "irradiated survivors."

Parisi and Antoine (8) previously reported that vegetative cells of this strain showed increased radiation resistance and permanent morphological and physiological changes after multiple radiation treatments. The extent to which a single substerilization process irradiation treatment affects spore nutrition, morphology, physiology, and radiation resistance has not been reported.

MATERIALS AND METHODS

Spore stock preparations. Spore stock suspensions of *B. pumilus* E601 (ATCC 27142) and all irradiated survivor strains resulting from this study were prepared by inoculating the surface of Trypticase soy agar (BBL) plates (25 ml in 15 by 150 mm plastic petri dishes) with 1.0 ml of a 24-h Trypticase soy broth (BBL) culture. The inoculated plates were incubated at 37 C until sporulation exceeded 95%. This usually occurred in less than 7 days. For strains which showed decreased sporulation ability, the agar medium was supplemented with 0.02% manganese sulfate. Spores were harvested by washing from the

agar surface with sterile deionized water followed by washing three times by centrifugation. The spores were then adjusted to the desired concentration.

Single dose irradiation and strain selection. Standard cotton suture (size 1 SUTUPAK COTTON, Ethicon, Inc., Somerville, N.J.) biological indicators were prepared by inoculating open vented 6-U strips of sutures with a spore suspension of *B. pumilus* E601 in 80% isopropyl alcohol-20% water to give a concentration of 2×10^4 spores per cotton suture biological indicator. After inoculation and air drying, the 6-U strips were heat-sealed and die-cut into individual suture packets. Five hundred of the suture biological indicators were exposed to 0.5 Mrad of ⁶⁰Co in a 10⁶-Ci source at Ethicon, Inc., Somerville, N.J. After irradiation treatment, the suture biological indicators were tested for sterility in Columbia broth (Difco) incubated for 7 days at 32 C. Tubes which showed less turbid growth than unirradiated controls, loss of pellicle growth were not selected for study. After and cells often growing in long filaments were selected as radiation-damaged strains. Tubes showing normal pellicle growth were not selected for study. After isolation, each survivor strain was routinely subcultured in Trypticase soy broth at 7-day intervals.

Gradient spore concentration and gradient irradiation. Paper strip biological indicators were prepared by inoculating Whatman no. 1 filter paper strips (1.25 by 2.5 cm) with 0.1-ml amounts of various levels of *B. pumilus* E601 spores in 80% isopropyl alcohol-20% water to give final concentrations of 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ spores. Fifty-six strips of each spore concentration were irradiated at doses of 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, and 1.75 Mrad with the same ⁶⁰Co source cited above. After irradiation, the strips were sterility tested and damaged strains were selected by the same method used for the suture indicators.

Determination of radiation resistance. The radiation resistance of each isolated strain showing radiation damage by the above criteria was deter-

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mined by irradiating 2.0-ml amounts of aqueous spore suspensions (1.0×10^7 per ml) in screw-cap vials (15 by 45 mm). For each survival curve, seven vials were prepared, with one serving as an untreated control and duplicates being irradiated with 0.5, 1.0, and 1.5 Mrads from a ^{60}Co source from a Gammacell 220 Irradiator (Atomic Energy of Canada, Ltd.) at the Central Research Laboratories of Johnson and Johnson, New Brunswick, N.J. Survival curves were obtained for each strain by plotting the surviving fractions of the initial population against irradiation dose, and the D_{10} value (the radiation dose required to reduce the initial population 1 log unit or 90%) was calculated. The best fit of the data to the curve was by the least squares method (3).

Characterization of strains. All strains selected as demonstrating radiation damage to be described in this study were characterized morphologically and physiologically using the procedure of Gordon et al. (4).

Nutritional studies. The nutritional requirements of spores of *B. pumilus* E601 and the irradiated survivor strains were determined by employing the procedures of White (10) and Knight and Proom (7). The basal medium contained (per liter): $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 10.4 g; KH_2PO_4 , 2.4 g; $(\text{NH}_4)_2\text{SO}_4$, 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.8 mg; and glucose, 10.0 g. The pH was 7.2 after sterilization. The basal medium minus the Mg^{2+} , Mn^{2+} , and Fe^{2+} salts and the carbon source was autoclaved for 10 min at 121 C. The salts, dissolved together at $50\times$ concentration in 0.01 N H_2SO_4 , and glucose as a 25% (wt/vol) aqueous solution were autoclaved separately.

Nutritional requirements for vitamins and trace metals were determined by adding supplements to the basal medium. Vitamins tested individually were (per liter): biotin, 1 μg ; folic acid, 2 μg ; pyridoxine hydrochloride, 0.5 mg; and calcium pantothenate, 0.5 mg. They were filter sterilized as $100\times$ aqueous solutions. Trace salts tested individually were (per liter): CaCl_2 , 1.0 mg; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 mg; H_3BO_3 , 0.1 mg; and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 mg. They were autoclaved as $100\times$ solutions in 0.01 N H_2SO_4 .

Individual amino acid requirements were determined by supplementing the complete basal medium with mixtures of various amino acids. Each complete assay medium lacked only a single amino acid. Stock solutions ($\times 50$) of the individual amino acids were used to make the 18 amino acid assay media and contained 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 aseptic addition of 5.0 ml of test medium to screw-cap (16 by 160 mm) (sterile) plastic disposable test tubes (Bio-

quest). Assays were performed in triplicate. Absence of growth at 72 h in a particular test medium was interpreted as indicating a requirement for the metabolite excluded from that medium. All media were made with 18-M Ω quality deionized water. All glassware for stock solutions was acid-cleaned and thoroughly rinsed in deionized water. Sterile disposable plastic pipettes and test tubes were used.

Spore inoculum for nutritional studies. Each spore suspension prepared according to the above procedure was washed an additional five times by centrifugation in sterile 18-M Ω deionized water, resuspended in sterile water, and adjusted to a concentration of about 10^6 spores per ml. These spore stocks were diluted to approximately 200 spores per ml and a test inoculum of 15 to 25 spores per 0.1 ml was verified by plate count on Trypticase soy agar, the inoculum used in the nutritional studies.

Vegetative cell inoculum for nutritional studies. Cells were grown in 100 ml of Trypticase soy broth on a gyrotary shaker at 32 C for 24 h. Cells were harvested by centrifugation, washed five times, and adjusted to a concentration of 2×10^6 cells per ml. Each tube was inoculated with 0.1 ml or 2×10^5 cells of this suspension.

RESULTS

Selection of strains. Of the 500 suture biological indicators irradiated with 0.5 Mrad of ^{60}Co , 16 (3.2%) produced cultures that showed morphological and cultural evidence of radiation damage. The alternative method used to produce damaged strains by the gradient spore-dose procedure produced only two visibly altered strains out of the 280 spore strips tested. Strain A-2 was recovered from a lowest spore concentration (10^5) paper strip from the lowest (0.25 Mrad) irradiation treatment and strain B-1 was recovered from a paper strip containing the highest spore level (10^9) tested and irradiated at the highest (1.25 Mrad) dosage. Designation of the 18 survivor strains is shown in Table 1. Although the criteria for strain selection were based on the initial outgrowth after irradiation treatment, the characteristics given in Table 1 are for survivor strains that were subcultured an additional six times prior to characterization.

Radiation resistance of sporulating strains. The D_{10} values of the sporulating parent and survivor strains are shown in Table 1 as a measure of their relative radiation resistances. The radiation resistance of the three strains (N-1, S-1, and T-1) which demonstrated less than 1% sporulation ability are not compared here. It will be noted that the D_{10} values ranged from 0.24 to 0.37 Mrad with the parent strain D_{10} value equaling 0.30 Mrad. Six strains demonstrated values lower than the parent and the values of six other strains were greater.

TABLE 1. Characterization of parent and irradiation-derived survivor strains of *Bacillus pumilus* E601^a

Determination	E601 ^a	A-2	B-1	C-2	D-1	E-2	F-1	H-1	I-1	J-1	K-2	M-1	N-1	P-1	P-2	Q-3	R-1	S-1	T-1
D ₁₀ (Mrad)	0.30	0.37	0.30	0.26	0.28	0.32	0.28	0.32	0.31	0.24	0.24	0.34	ND	0.28	0.30	0.30	0.34	ND	ND
Spores formed	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Motility	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+
Pellicle type	Std	Std	Cm	Std	Th	Cm	Sm	Std	Th	Std	Std	-	-	Std	Std	Th	Std	-	Sm
Anaerobic growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maximal growth temp. (C)	44	44	44	44	44	44	44	44	40	44	44	44	40	44	44	40	44	40	44
Starch hydrolyzed	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduced	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilized	+	+	Wk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate utilized	+	+	Wk	+	+	+	+	+	+	+	+	+	+	+	Wk	+	+	+	+
Catalase produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetoin produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dihydroxyacetone produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein decomposed	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Glucose fermented	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Trehalose fermented	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Mannitol fermented	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Arabinose fermented	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Growth, 5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth, 7% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth, pH 5.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Final pH, glucose broth	4.7	4.5	4.8	4.8	4.5	4.5	4.5	4.6	4.3	4.4	4.6	4.8	4.7	4.6	4.5	4.8	4.8	4.9	4.8

^a All tests were run in triplicate. Abbreviations: +, positive; -, negative; ND, not done; Std, standard; Cm, creamy; Th, thin; Sm, smooth; Wk, weak; and A, acid.

^b Unirradiated parent.

Characteristics of strains. Table 1 also compares the morphological and physiological characteristics of the 18 survivor strains with the parent strain from which they were derived. Of the 18 strains, eight maintained normal pellicle-forming ability and three formed thinly veiled pellicles. Other traits altered or lost included: reductions in maximal temperature of growth (four strains), little or no production of catalase (four strains), no production of acetoin (two strains), no growth in 7% NaCl (five strains), and one sugar (trehalose) fermentation change.

Nutritional requirements for spore germination and outgrowth. The nutritional requirements of the sporulating survivor strains are shown in Table 2. Spores of all strains, including the parent strain, were found to require the usual carbon and nitrogen sources, biotin, and at least one amino acid for germination and outgrowth. No requirements were found for the other vitamins and trace metals tested.

The parent strain spores required the single amino acid, valine. Only one survivor strain (B-1) showed a similar singular requirement. The number of amino acids required for the other strains ranged from four (strain I-1) to 15 (strain A-2). The frequency with which a particular amino acid was required by the collection of survivor strains also varied. In addition to the valine requirement exhibited by all spores, it was found that aspartic acid (14 strains), threonine (14 strains), isoleucine (13 strains), and glutamic acid (12 strains) were most frequently required for survivor strain spore germination and outgrowth. Proline was the single amino

acid for which a requirement was never demonstrated. The frequency of requirement for the remaining amino acids was considerably less than these amino acids.

Nutritional requirements of the nonsporulating strains. The vegetative cell nutritional requirements of the three strains (N-1, S-1, and T-1) which did not readily sporulate are given in Table 3, using the same test procedure employed for the nutritional requirements of the sporulating strains. The parent strain vegetative cells were found competent to synthesize all amino acids from glucose and ammonium when biotin was supplied as a cofactor. The asporogenous survivor strains, however, were shown to have requirements involving five different amino acids with aspartic acid and/or lysine being required by the strains. It was of interest that valine, the amino acid generally essential for spore germination, was required by the S-1 strain for vegetative growth.

TABLE 3. Nutritional requirements of parent and ^{60}CO irradiation-derived asporogenous vegetative strains of *Bacillus pumilus* E601^a

Strain	Requirements		
	NH ₄ ⁺ , glucose, salts	Vitamins	Amino acids
Parent	+	Biotin	None
N-1	+	Biotin	Asp, Lys
S-1	+	Biotin	Asp, Val
T-1	+	Biotin	Lys, Ile, Thr

^a Tests were run in triplicate and read after 72 h.

TABLE 2. Amino acid requirements for spore germination and outgrowth of irradiation-derived survivor strains of *Bacillus pumilus* E601 relative to radiation resistance^a

Strain	D ₁₀ (Mrad)	Amino acid required																	
		Ala	Asp	Glu	Pro	Met	Arg	Gly	Ser	Phe	Try	Tyr	His	Ile	Leu	Thr	Val	Cys	Lys
J-1	0.24	+	+	+	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+
K-2	0.24	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-
C-2	0.26	-	+	+	-	-	-	-	-	+	+	-	+	+	-	+	+	-	+
D-1	0.28	+	+	+	-	-	+	-	-	+	+	-	-	-	-	+	+	+	-
F-1	0.28	-	+	+	-	+	-	-	-	+	+	-	-	+	-	+	+	+	-
P-1	0.28	+	+	+	-	-	-	-	+	+	-	-	+	+	-	+	+	+	+
B-1	0.30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
P-2	0.30	+	+	+	-	-	-	-	+	+	-	-	+	+	-	+	+	+	+
Q-3	0.30	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	+	-	+
I-1	0.31	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-
H-1	0.32	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-
E-2	0.32	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-
M-1	0.34	+	+	+	-	+	-	-	+	+	-	-	-	+	-	+	+	-	+
R-1	0.34	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-
A-2	0.37	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+

^a Positive (+) or negative (-) spore germination and outgrowth after 72 h. Tests were run in triplicate.

Although not shown, the amino acid requirements for the vegetative cell growth of the sporulating survivor strains were minimal when compared to the requirements for spore germination and outgrowth shown previously in Table 2. Finally, aside from the requirements for biotin and certain amino acids for vegetative cell growth, no requirements were found for any other vitamins and trace metals tested.

DISCUSSION

We previously reported (8) that *B. pumilus* E601 spores which survived irradiation treatment, high dose or low dose, frequently showed evidence of radiation damage. This injury was manifested in typical elongated filamentous cellular forms having impaired cross wall formation. When compared to the parent strain, the radiation-damaged cells generally demonstrated a loss of motility and were further characterized for their inability to form thick pellicles which are typical of the parent strain. Selection of the 18 irradiation treatment survivor strains of *B. pumilus* E601 described in the present study was based primarily upon cultural and morphological deviations from the parent strain characters, i.e., loss of motility and pellicle formation. With subsequent subcultivation after the initial selection, however, a number of the survivor strains reverted back to the standard, exhibiting motility and forming pellicles, although the majority of the strains remained visibly altered. As a result, 10 survivor strains are reported that did not form pellicles or did so sparingly after irradiation treatment and subsequent subcultivation. These radiation-damaged strains consistently demonstrated quantitatively decreased growth compared to the pellicle-forming parent strain. Five of these nonpellicle-forming strains remained nonmotile. The loss of motility and concurrent decrease in pellicle formation in these strains is presumably due to their inability to migrate to the site of maximal oxygen concentration at the surface of the culture medium. In addition, eight strains are described which, upon repeated subcultivation, were morphologically indistinguishable from the parent strain with characteristic motility and pellicle-forming ability but with other altered physiological characteristics and radiation resistance.

All survivor strains were produced by sublethal ^{60}Co treatment of parent strain spores. The most successful method of producing damaged cultures was effected by a single ^{60}Co irradiation treatment at the highest sublethal ^{60}Co dosage. It would appear that the greatest radiation damage, at least in terms of morphological alteration, was produced in three survivor

strains which were found to be asporogenous upon isolation and after repeated subcultivation. Asporogenous mutants of the closely related species *B. subtilis* have been reported in the literature, and the mutation has been shown to be the result of a deletion of a segment of deoxyribonucleic acid along the phe-1-lys-1 region of the chromosome (1, 6) and located in the terminal portion of the chromosome (5).

All physiological alterations detected in the 18 substrains were evidenced by the apparent loss of some enzymatic functions resulting in the inability to carry out a reaction as in the parent strain. The radiation resistances (D values) of the spore forming substrains were spread over a relatively narrow range (0.24 to 0.37 Mrad) which apparently is a reflection of the range of resistance of the individual members of the parent strain normal population. The single sublethal radiation treatment produced no unusual alteration (increase or decrease in resistance) compared with the parent. This is also in agreement with that reported earlier for this *B. pumilus* strain after a single exposure to beta particles from a cathode ray tube in which a single survivor strain was examined (9). The survivor strain D values are also consistent with those reported for other species of sporeformers (2). All survival curves were straightline logarithmic regressions with no evidence of the often typical shoulder at the lower radiation doses. The configuration of the irradiated strain survival curves is consistent with that of the parent strain.

No correlations could be shown between strains with high radiation resistance and those which demonstrated more exacting nutritional requirements than the parent strain for spore germination and outgrowth. Of the strains examined, only one showed no evidence of alteration of nutritional requirements compared with that of the parent, which needed only valine in addition to its vegetative cell requirements for outgrowth from the spore state.

Enzymes essential to the biosynthesis of the amino acids of the aspartate family which have oxaloacetate as the common precursor were apparently selectively sensitive to the radiation treatment. In the sporeforming strains, requirements were most common for aspartic acid, threonine, and isoleucine. Glutamic acid as a member of the glutamate family of amino acid synthesis which has α -ketoglutaric acid as the precursor was somewhat less frequently required by the sporulating survivor strains. The asporogenous strains exhibited relatively few amino acid requirements, with aspartic acid and/or lysine most frequently required.

The appearance of biological indicator survi-

vors are extremely rare after the normal 2.5-Mrad sterilization dosage and the nutritional requirements of these survivors are uncertain. The survivor strains isolated in this study can serve as valid representatives of the effects of gamma radiation on the biological indicator spore. The only nutritional requirements produced by the single sublethal gamma irradiation treatments were for amino acids which are normally supplied by commercial sterility test media. These new requirements in no way affect the value of the parent strain, *B. pumilus* E601, as a biological indicator and, in fact, provide significant information relative to the behavior of this strain upon irradiation.

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