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# Influence of Postirradiation Incubation Temperature on Recovery of Radiation-Injured *Clostridium botulinum* 62A Spores

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The number of colonies formed by unirradiated *Clostridium botulinum* 62A spores was independent of temperature, in the range from 20 to 45°C (in 5°C increments); no colonies developed at 50°C. Spores irradiated at 1.2 or 1.4 Mrads produced more macrocolonies at 40°C than at higher or lower temperatures. Apparently, radiation-injured spores were more capable of repair at 40°C than at the other temperatures studied. More than 99% of the radiation (1.2 Mrads) survivors were injured and were unable to form macrocolonies in the presence of 5% NaCl. The germinated radiation-injured spores were also sensitive to dilution, resulting in the loss of viability of 77 to 79% of the radiation survivors. At 30 and 40°C, the irradiated spores did not differ significantly in the extent of germination (>99% at both 30 and 40°C), emergence (64% at 30°C and 67% at 40°C), and in the maximum number of emerged cells that started to elongate (69% at 30°C and 79% at 40°C). However, elongation was remarkably more extensive at 40°C than at 30°C. Many elongated cells lysed within 48 h at 30°C, indicating an impaired repair mechanism. If the radiation-injured spores were incubated at 40°C in the recovery (repair) medium for 8 to 10 h, they germinated, emerged, and elongated extensively and were capable of repair. If, after 8 to 10 h at 40°C, these cultures were shifted to 30°C, the recovery at 30°C increased by more than eightfold, resulting in similar colony counts at 30 and 40°C. Thus, repair appeared to be associated with outgrowth. Repair did not occur in the presence of chloramphenicol at 40°C, whereas penicillin had no effect, suggesting that the repair involved protein synthesis but did not require multiplication.

Exposure of bacteria to substerilizing doses of irradiation or heat produced cell injury, and the injured population could be quantitatively measured by the use of sodium chloride (12, 13) in the recovery medium, injured cells not growing in the presence of NaCl (20).

The temperature of incubation influenced the recovery of heat-injured spores. The recovery of heat-damaged *Clostridium botulinum* spores was better at suboptimal than at more optimal growth temperatures of 31 or 37°C (15, 24, 27). Heated *Bacillus subtilis* spores recovered better at 32°C than at 45°C (8) and better at 30°C than at higher or lower temperatures (16).

Fewer reports are available on the recovery of radiation-damaged *C. botulinum* spores. Better recovery of radiation-damaged *C. perfringens* spores was obtained at 21°C than at higher temperatures (10). In a study with *C. botulinum* type A (NCTC 3806), Roberts (19) reported that the recovery of radiation (0.43 Mrad)-damaged spores was 11-fold greater at 35°C than at higher or lower temperatures. The recovery at 30 and 40°C was the same. The recovery of irradiated (0.5 Mrad) spores of *C. botulinum* type B was twofold greater at 37°C than at 30°C (26).

Although the best recovery of heat-damaged spores tends to be at suboptimal growth temperatures, the best temperatures for recovery of radiation-damaged spores have not been characterized and this clearly needs further study. The purpose of this investigation was to determine the influence of incubation temperature on the recovery of radiation-injured *C. botulinum* 62A spores.

(Some of the data in this paper were presented at the 75th Annual Meeting, American Society for Microbiology, New York, N.Y., 27 April to 2 May 1975.)

## MATERIALS AND METHODS

**Preparation of spore stock.** Spores of *C. botulinum* 62A, produced by a biphasic culture system using 2% $(NH_4)_2SO_4$ as the liquid phase (6), were

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washed 10 times by suspending in sterile, distilled water and centrifuging for 5 min at 4,080 × g and 5°C). The pellet was cleaned of sporangia, vegetative cells, and debris by the method of Rowley and Feeherry (21), except that the trypsin-lysozyme mixture was adjusted to pH 6.0 instead of pH 8.1, since at pH 8.1 some activation of the spores occurred (Rowley and Feeherry, unpublished data). The cleaned spores (96 to 97% phase bright) were resuspended (about $1 \times 10^9$/ml) in distilled water, dispensed in 5-ml lots in screw-cap tubes, frozen quickly with acetone-dry ice, and stored at −30°C. Spores were thawed at 4°C and stored at this temperature for use within 7 days.

**Irradiation of spores.** For the determination of survival alone, $10^7$ spores/ml of distilled water were heated at 80°C for 10 min to inactivate any vegetative forms. When studying germination, $>3 \times 10^8$ spores/ml of distilled water were heat activated at 80°C for 60 min (21). In either case, after heating, spore suspensions were rapidly cooled in an ice bath, vacuum sealed (125 mm of Hg) in Pyrex tubes (16 by 150 mm), quickly frozen in acetone-dry ice, equilibrated overnight at −30°C, and irradiated at −30 ± 5°C in a $^{60}$Co gamma source. The dose rate was 13,917 rads/min, and the transient dose was 1,200 rads.

**Determination of survivors.** Survival was determined by counting macrocolonies in deep agar tubes (11 by 202 mm) after incubation at 20 to 55°C in 5°C increments for 1 to 36 days.

**Recovery (repair) medium.** The medium (TYT) for counting macrocolonies consisted of 5% Thiotone (BBL), 0.5% yeast extract (BBL), 0.5% Trypticase (BBL), 0.05% sodium thioglycolate (BBL), 0.75% agar (Difco), and 0.075% $NaHCO_3$. The medium, without agar and $NaHCO_3$, was adjusted to pH 7.2 with 5 N NaOH and autoclaved after the addition of agar. The $NaHCO_3$ (5% stock) was filter sterilized and added (0.15 ml) to the count tubes prior to inoculation and addition of the recovery medium to a final volume of 10 ml. TYT broth contained all of the above ingredients except agar.

**Spore germination and survival of germinated spores.** TYT broth in standardized Klett colorimeter tubes was steamed for 20 min and cooled to the desired temperature (30 or 40°C), and pretempered $NaHCO_3$ and spores were added. The Klett tubes, containing a final volume of 10 ml ($10^8$ spores/ml), were plugged with serum stoppers, and the headspace (4.5 ml) was flushed with sterile nitrogen (99.997% pure) for 2 min and quickly placed in a water bath at the desired temperature.

**Estimation of germination, emergence, and elongation.** Stages of development were followed by microscope examination using Zeiss dark contrast-phase optics.

**Determination of radiation-injured population. (i) Sensitivity to NaCl.** Unirradiated spores and spores uninjured by irradiation form colonies on TYT agar in the presence or in the absence of 5% NaCl. Certain radiation-injured spores produced colonies in the absence of NaCl but not in its presence, and the difference in colony counts with and without NaCl was used as a measure of injury.

**(ii) Sensitivity to dilution.** At various stages of germination and outgrowth, 0.1 ml of the suspension was withdrawn with a sterile, disposable hypodermic syringe and serially diluted preparatory to counting macrocolonies in TYT agar. Many diluents tested (e.g., distilled water, 0.05% sodium thioglycolate, one-half-strength TYT broth plus 0.05% sodium thioglycolate) effectively reduced the colony count of unirradiated spores undergoing germination and outgrowth. This reduction in count did not occur when suspensions of the germinating or outgrowing spores were diluted in half-strength TYT broth containing 0.5% sodium thioglycolate (M. S. U. Chowdhury, D. B. Rowley, and A. Anellis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I143, p. 140), and this was the diluent used in the experiments reported here. A reduction in the count of germinating irradiated spores, in the absence of such a reduction in count of unirradiated spores, may be considered as a manifestation of injury to the radiation survivors.

**Microculture.** Microculture was the technique applied to the determination of germinability and morphology of irradiated spores during outgrowth at 30 and 40°C. The method of Duncan and Foster (7) was adopted, using a slide (50 by 75 mm) and cover slip (22 by 40 mm). TYT agar was used as the medium. The slide cultures were examined at intervals with phase-contrast optics. Photographs were taken periodically using Polaroid 4- by 5-inch Land film (black and white).

The microculture technique was also used to determine the minimum concentration of inhibitors that prevented the elongation or multiplication of cells after emergence from spores.

**Metabolic inhibitors.** In the presence of chloramphenicol (100 μg/ml), unirradiated *C. botulinum* spores germinated but did not elongate and form macrocolonies. Penicillin (0.25 U/ml) also allowed germination and prevented the formation of macrocolonies. However, unlike chloramphenicol, penicillin allowed some elongation (10 to 15%/10 h). Irradiated spores were appropriately diluted in TYT broth with chloramphenicol (100 μg/ml) or penicillin (0.25 U/ml), and 0.1 ml was dispensed in cotton-plugged deep-counting tubes and incubated in an anaerobic jar. After 10 h of incubation at 40°C, TYT agar was added to give a final volume of 10 ml and, after solidification, was layered with TYT agar to insure anaerobiosis. The concentrations of chloramphenicol and penicillin in the final counting tubes (1 μg/ml and 0.0025 U/ml, respectively) did not affect the germination, outgrowth, and macrocolony formation of spores.

## RESULTS

**Recovery at various temperatures.** Colony counts of unirradiated spores were generally independent of temperature over the range 20 to 45°C; no macrocolonies were formed at 50 and 55°C, even after 36 days of incubation (Fig. 1). The range of incubation temperatures giving maximum recovery of irradiated spores narrowed as the dose increased until, after 1.2 and 1.4 Mrads, 40°C was clearly optimal for macro-

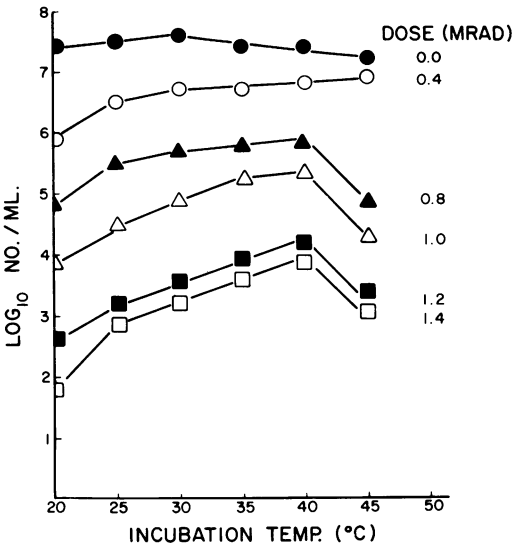


FIG. 1. *Influence of incubation temperature on colony counts of radiation-damaged C. botulinum 62A spores. Spores were heat shocked at 80°C for 10 min, cooled, vacuum sealed, frozen, irradiated at −30 ± 5°C, and recovered in TYT agar (see text).*

colony formation. The difference in the number of colonies between any two neighboring temperatures of spores irradiated at 1.2 and 1.4 Mrads was significant at the 95% confidence level. Holding count tubes at 4°C for various periods of time before incubation at 30 or 40°C did not influence colony counts at these temperatures.

**Sensitivity to NaCl.** The number of radiation survivors (0.01%) was greatly reduced when 5% NaCl (wt/vol) was incorporated with TYT agar, whereas the count of unirradiated spores remained unaffected. This sensitivity of radiation survivors to 5% NaCl was a measure of injury in the irradiated sample. More than 99% of the spores surviving 1.2 Mrads were injured, being unable to form macrocolonies in the presence of 5% NaCl (Table 1).

**Sensitivity to dilution.** One-half-strength TYT broth containing 0.5% sodium thioglycolate was used as a diluent for recovering ungerminated and germinated spores. Germination did not affect the colony count of unirradiated spores (45 to 50% germination, 100% recovery; 97 to 98% germination, 92% recovery) but did markedly reduce the colony count of irradiated spores (Table 2). During the germination of *C. botulinum* 62A spores, there is a definite sequence of events (21). In radiation-damaged (but not in unirradiated) spores, sensitivity to dilution may be one of these events. This sensitivity may (and, indeed, probably does) occur before phase darkening and stainability. From Table 2, it seems possible that phase-dark (germinated) spores were already sensitive to dilution. Indeed, by the time 45 to 50% of the irradiated spores had germinated, the germination event involving sensitivity to dilution was already completed and did not increase further, in spite of an increasing percentage of germination.

**Characterization of outgrowth of radiation-injured spores.** As determined by the microculture technique, irradiated (1.2 Mrads) spores did not differ at 30 and 40°C in the extent of germination (99% phase dark and stainable at both 30 and 40°C), emergence (64% at 30°C and 67% at 40°C), and perhaps in the number of emerged cells that elongated (69% at 30°C and 79% at 40°C). However, the extent of elongation of irradiated cells was substantially different at 40 and 30°C. At 40°C (Fig. 2A to D) and also at 30°C (data not shown), the unirradiated spores germinated, outgrew, and divided normally within 5 h and formed microcolonies by 10 h without filament formation. At 40°C, irradiated spores elongated rapidly and extensively and produced aseptate filaments by 10 h (Fig. 2E). These filaments, about 15 to 20 times longer than the typical vegetative cell, on further incubation divided into smaller cells (Fig. 2F),

TABLE 1. *Inactivation and injury of irradiated C. botulinum 62A spores*[a]

| NaCl (%) | Recovery of: | | Inactivation (%) | Injury (%) |
|---|---|---|---|---|
| | Unirradiated spores[b] | Irradiated spores[b] | | |
| 0 | $2.3 \times 10^7$ | $2.6 \times 10^3$ | 99.99 | |
| 5 | $2.4 \times 10^7$ | $1.2 \times 10^1$ | | 99.54 |

[a] Spores for irradiation (1.2 Mrads) were prepared as noted in the Legend to Fig. 1. Macrocolony formation was determined with TYT agar and TYT agar plus 5% NaCl. Incubation was at 30°C for 1 to 14 days.
[b] Number of colonies per milliliter.

TABLE 2. *Sensitivity of radiation (1.2 Mrads)-injured C. botulinum 62A spores to dilution after germination at 30°C in TYT broth*[a]

| Germination (%) | Germination time (h) | Recovery of: | |
|---|---|---|---|
| | | Unirradiated spores | Irradiated spores |
| 0 | 0 | $1.2 \times 10^{8}$[b] (100)[c] | $1.6 \times 10^{4}$[b] (100)[c] |
| 45–50 | 1 | $1.2 \times 10^{8}$ (100) | $3.6 \times 10^{3}$ (23) |
| 97–98 | 2 | $1.1 \times 10^{8}$ (92) | $3.4 \times 10^{3}$ (21) |

[a] Spores were germinated at 30°C in TYT broth, and samples were diluted periodically in one-half-strength TYT broth containing 0.5% sodium thioglycolate. Recovery was determined in deep agar tubes using TYT agar.
[b] Number of colonies per milliliter.
[c] Percentage of initial count.

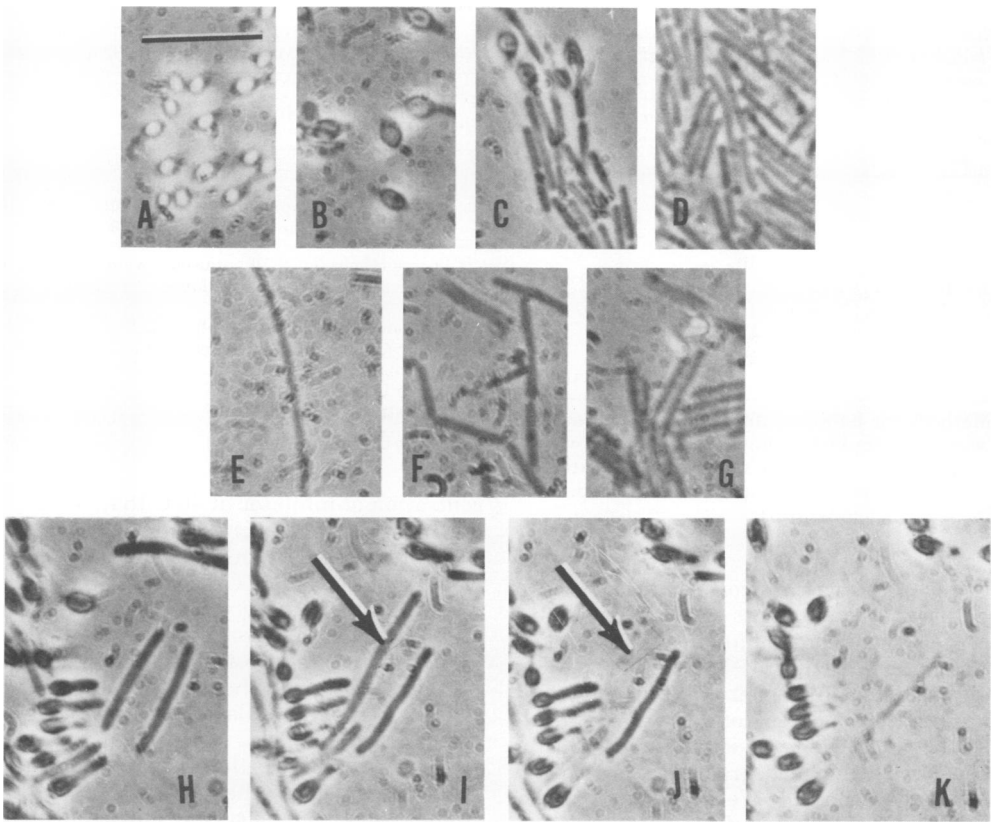


FIG. 2. *Influence of incubation temperature on postgerminative development of C. botulinum 62A spores in microculture. (A to D) Unirradiated spores incubated at 40°C. (A) Dormant phase-bright spores; (B) germinated and swollen spores and emerging cells, 3 h; (C) division of elongated cells, 5 h; (D) microcolony, 10 h; (E to G) irradiated (1.2 Mrads) spores incubated at 40°C; (E) portion (15 to 20%) of a typical filamentous cell, 10 h; (F) dividing filament, 13 h; (G) portion of microcolony, 19 h; (H to K) irradiated (1.2 Mrads) spores incubated at 30°C (all same field); (H) elongated cells, 10 h; (I) elongated cells, 25 h, with little apparent increase in growth; (J) beginning of lysis, 32 h (note cell indicated by arrow in I and J); (K) lysis of most emerged and elongated cells, 48 h. Bar represents 10 μm.*

which eventually divided to form microcolonies within 19 h (Fig. 2G). Irradiated spores, incubated at 30°C, showed only limited elongation by 10 h (Fig. 2H) and remained apparently stunted even by 25 h (Fig. 2I). On further incubation, the cells began to lyse within 32 h instead of producing vegetative outgrowth (Fig. 2J), and by 48 h many of the cells had lysed (Fig. 2K). This indicated a lack of repair and growth of the emerged cells at 30°C, whereas most of such cells grew rapidly and extensively at 40°C.

**Influence of preincubation of radiation-injured spores at 40°C.** At 40°C more radiation-injured spores were capable of recovery and producing macrocolonies than at 30°C. To determine during what stage of germination and outgrowth at 40°C the recovery took place, irradiated spores were diluted, tubed, and incubated at 40°C for various time intervals before shifting the count tubes to 30°C.

The colony count at 30°C was not increased as the result of prior incubation for 2 h at 40°C (Table 3). During this same period at 40°C, ≥98% of the spores had germinated, suggesting that repair of injury did not take place during germination. After 4 h of incubation at 40°C, the colony counts began to increase at 30°C. Radiation-injured spores had undergone repair within 8 to 10 h of incubation at 40°C, the time interval during which the aseptate filaments formed, so that when shifted to 30°C after this period the final count was equivalent to that obtained by 28 h of incubation at 40°C.

**Effect of pantoyl lactone.** Adler and Hardigree (1) observed that irradiation produced mu-

TABLE 3. *Effect of incubation of radiation-injured C. botulinum 62A spores in the repair (recovery) medium at 40°C on their survival at 30°C*[a]

| Time (h) of preincubation at 40°C | Colony counts/ml at: | |
|---|---|---|
| | 30°C[b] | 40°C |
| 0 | $5.5 \times 10^4$ | |
| 1 | $5.4 \times 10^4$ | |
| 2 | $5.7 \times 10^4$ | |
| 4 | $1.2 \times 10^5$ | |
| 6 | $2.0 \times 10^5$ | |
| 8 | $4.0 \times 10^5$ | |
| 10 | $4.6 \times 10^5$ | |
| 12 | $4.6 \times 10^5$ | |
| 28 | | $4.5 \times 10^5$ |

[a] Methods for handling and counting spore samples were as described in the legend to Fig. 1, except that spores ($1.3 \times 10^8$/ml) were heat activated at 80°C for 60 min prior to irradiation (1.2 Mrads).
[b] Incubation temperature.

TABLE 4. *Effect of inhibitors on the repair of radiation injury of C. botulinum 62A spores*[a]

| Preincubation[b] | Inhibitor[c] | Colony counts/ml at: | |
|---|---|---|---|
| | | 40°C[d] | 30°C |
| 0 | None | $7.4 \times 10^6$ | $6.2 \times 10^5$ |
| 10 | None | $9.0 \times 10^6$ | $9.1 \times 10^6$ |
| 10 | Chloramphenicol | $6.0 \times 10^3$ | $8.8 \times 10^2$ |
| 10 | Penicillin | $9.1 \times 10^4$ | $9.4 \times 10^4$ |

[a] Spores (about $9.0 \times 10^9$, aqueous) were heat activated at 80°C for 60 min prior to irradiation (1.2 Mrads).
[b] Hours at 40°C.
[c] Chloramphenicol, 100 μg/ml; penicillin, 0.25 U/ml. In the final counting tube, these concentrations were diluted to noninhibitory levels, i.e., 1 μg of chloramphenicol/ml and 0.0025 U of penicillin/ml.
[d] Incubation temperature.

tant cells of *Escherichia coli* K-12 that formed long nonseptate filaments when grown after exposure to ionizing radiation. The filaments did not give rise to macrocolonies. The addition of pantoyl lactone (an agent that initiates cross-plate formation) to the complete medium allowed the filaments to divide and form macrocolonies, resulting in a great increase in the survival of the mutant. We added pantoyl lactone to TYT agar to yield final concentrations from 0.05 to 0.2 M, but this agent had no effect at either 30 or 40°C on the number of macrocolonies produced (data not shown). Pantoyl lactone was also added to TYT broth after the irradiated spores had outgrown at 40°C (8 h); microscope observations did not reveal any induction of cross wall formation in the filaments; i.e., no difference between control (without pantoyl lactone) and test samples could be detected during 2 h of observation. This was also true at 30°C. These results suggest that the repair of radiation injury in *C. botulinum* 62A spores was not related to the ability of cells to form cross walls.

**Effect of metabolic inhibitors.** The irradiated (1.2 Mrads) spores produced approximately 12-fold more macrocolonies at 40°C than at 30°C when no preincubation (10 h) was used before shifting to 30°C and no inhibitor was present in the recovery medium (Table 4). Similar colony counts at 40 and 30°C were obtained if the irradiated spores were first incubated for 10 h at 40°C. If radiation-injured (capable of forming macrocolonies at 40°C but not at 30°C) spores were (i) incubated for 10 h at 40°C in the presence of a metabolic inhibitor of repair synthesis, there should be more colonies developing on further incubation with a 100-fold reduced concentration of inhibitor at 40°C than at 30°C. If, on the other hand, (ii) the added chemical did not inhibit repair synthesis, as many colonies would develop at 30°C as at 40°C. Chloramphenicol falls in category (i), with seven times as many macrocolonies developing at 40°C as at 30°C (Table 4), suggesting the involvement of protein synthesis in the repair of this radiation injury. Penicillin falls in category (ii), with colony counts at 30°C equaling those at 40°C (Table 4), indicating that penicillin did not inhibit this repair synthesis.

The reduction in survivors during incubation with chloramphenicol or penicillin (Table 4) may have been due to the sensitivity of germinated spores, as in the case of dilution (Table 2), and may have represented yet another manifestation of radiation injury. This aspect was not further investigated.

## DISCUSSION

The composition of recovery media influenced the survival of radiation-damaged vegetative cells (3, 4, 18) and spores (17). In our investigation, no attempt was made to determine whether TYT agar and broth were optimal substrates for the repair of radiation-injured spores. However, this medium has been used over the years in this laboratory and is equally satisfactory for macrocolony counts of unirradiated and irradiated spores of many strains of *C. botulinum*.

Incubation temperature affected the ability of irradiated vegetative cells (5, 22) or spores (10, 19, 26) to form macrocolonies. Our results (incubation at 40°C was optimal for macrocolony development of survivors after a severely damaging gamma irradiation treatment of *C. botulinum* 62A spores) generally agree with those reported for radiation-damaged *C. sporogenes* (PA 3679) and *C. botulinum* type B (26)

and *C. botulinum* type A, NCTC 3806 (19). The report that severely radiation-damaged (0.01% survival) *C. perfringens* spores recovered better at 21°C than at higher temperatures (10) contrasts markedly with our findings for *C. botulinum* 62A. Such differences suggest that optimum recovery temperatures of damaged spores may be species or strain dependent. Such variations have also been shown for different strains of vegetative cells (2, 23).

Maintaining radiation-injured *E. coli* B/r for 24 h at a suboptimal temperature (18°C) for growth allowed partial recovery from the lethal effects of X irradiation (23), the repair of injury perhaps involving an active physiological process. Lowering the postradiation temperature to 0 to 2°C for 24 h did not alter the survival of *B. pumilus* spores at 20 to 25°C (9), nor in our study did holding irradiated (1.2 Mrads) spores at 4°C in TYT recovery agar up to 2 weeks alter colony counts at 20, 30, and 40°C. At 4°C, the injured spores were metabolically inactive and, therefore, no repair of radiation injury could take place.

That spores were indeed injured by radiation was indicated by three criteria: (i) sensitivity to NaCl, (ii) sensitivity to dilution after germination, and (iii) inability to form macrocolonies at 30°C. There was no evidence that each criterion was an indication of injury of the same nature or extent. In this study, we were concerned with the injured cells capable of forming macrocolonies at 40°C but not at 30°C. The cells giving rise to filamentous forms at 40°C and those lysing at 30°C were the injured ones, since uninjured cells emerged, elongated, and divided without filament formation or lysis (Fig. 2A to D). Conceivably, the temperature effect (8- to 12-fold more colonies at 40°C than at 30°C) involved repair of the damaged spores and took place at some stage(s) after germination and during outgrowth, resulting in macrocolony formation; some metabolic or physiological process(es) may have been more operative at 40°C than at 30°C. It seems evident, indeed, that radiation-injured spores were fully as capable of germination (and perhaps of emergence) as were unirradiated spores; the injury affected the capacity for further outgrowth and cell division. It was this capacity for outgrowth and cell division that was repaired at 40°C. Prentice and Clegg (16) also postulated that the influence of temperature on the recovery of heat-treated *B. subtilis* spores occurred at some stage other than germination and probably during outgrowth.

The repair of injury that took place at 40°C in 10 h was inhibited by chloramphenicol but not by penicillin (Table 4), suggesting that repair was dependent on protein synthesis and occurred prior to the septation stage. A requirement for protein synthesis was reported for the repair of thermally injured *Salmonella typhimurium* (25) but not for *Staphylococcus aureus* (13), *Pseudomonas fluorescens* (11), or *B. subtilis* (14).

No attempt was made to elaborate further the repair synthesis. It is evident, however, that the repair process is more operative at 40°C than at 30°C, involves protein synthesis, and occurs during outgrowth and prior to cell division. The temperature of postirradiation incubation should be considered in determining the survival and/or recovery of radiation-damaged *C. botulinum* 62A spores or in determining the sterility of irradiated products.

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