Repair of Heat-Injured Clostridium perfringens Spores During Outgrowth

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Clostridium perfringens strain NCTC 8798 spores were injured by ultrahigh temperature treatment and were unable to outgrow in the presence of antibiotics used in selective enumeration media. Injured spores underwent repair in a nonselective laboratory medium and in foods.

Recent reports have indicated that heat treatment of Clostridium perfringens spores may result in (i) dead spores, (ii) injured survivors, and (iii) uninjured survivors (1–5). Some of the injured spores may have damaged germination systems and require lysozyme for germination (5). Injured spores also have been shown to be more sensitive than uninjured spores to nitrite, NaCl, and certain antibiotics (3, 6, 8), probably because of damaged outgrowth systems (6). Under suitable conditions, injured spores may repair and behave as normal spores. The increased sensitivity of ultrahigh temperature (UHT)-treated C. perfringens spores to polymyxin and neomycin and the repair of this injury were investigated.

Preparation of C. perfringens type A strain NCTC 8798 spore suspensions, culture maintenance, and enumeration procedures have been described (1). UHT-treated spores were enumerated on tryptone-sulfite-neomycin agar (TSN) (7) and TSN lacking antibiotics (BASE). UHT-treated spores were sensitized to lysozyme by incubating them in 0.1 N NaOH (pH 13) at 45 °C for 15 min; they were enumerated on BASE and TSN, both containing 1 μg of lysozyme per ml (18,000 U/mg; Sigma Chemical Co., St. Louis, Mo.). Repair occurred in BASE broth (BASE without agar) and BASE broth with lysozyme. Milk or meat broth was prepared as described (3). The number of colony-forming units (CFU) or growth in most-probable-number tubes was determined after anaerobic (90% N2-10% CO2) incubation at 35 or 45 °C for 24 h. Filter-sterilized (Millipore Corp., Bedford, Mass.) antibiotics (Sigma) or lysozyme or both were added to TSN broth (TSN without agar), most-probable-number tubes, and to the melted and cooled (45 °C) TSN just prior to plating.

The repair of heat-injured spores is shown in Fig. 1. Initially, the number of CFU of UHT-treated spores on TSN were less than 1% of the number on BASE. Differences in the number of CFU on selective and nonselective media suggested 99.8% of the survivors were injured. Only uninjured spores formed colonies on TSN. Repair of injured spores in BASE broth at 45 °C was indicated by regained capacity to form colonies on TSN. Repair lagged behind germination by up to 2 h, and 20% repair occurred prior to the onset of growth (3 h). Repair of injured spores beyond 3 h was masked by growth of uninjured or repaired spores.

To distinguish the repair of injured spores from the growth of uninjured spores, the time required for germination, outgrowth, and growth of non-UHT-treated spores was determined. At least 2 h elapsed before uninjured spores started growth, whereas repair started almost immediately. This indicated that repair occurred during outgrowth and suggested that the UHT treatment damaged a spore structure or metabolic system involved in normal outgrowth. Labbe and Duncan (6) also found that injured C. perfringens spores sensitive to sodium nitrite germinated but did not outgrow. Injured spores apparently varied in the extent of thermal damage because the time required for repair varied greatly.

The majority of C. perfringens spores that survived UHT treatment could not germinate normally (1, 5) and were not detected in the previous experiment. To determine if these spores also suffered repairable damage to the outgrowth system required that the spores be sensitized to and germinated by lysozyme (2). Treatment of the survivors with alkali followed by germination with lysozyme was effective for studying repair of these spores. The time-survivor curve for spores heated at 105 °C and treated with alkali prior to plating on BASE plus lysozyme was linear, unbroken, and extrapolated to an ordinate intercept equal to the number of viable spores prior to UHT treatment (Fig. 2). This indicated that the alkali-lysozyme treatment permitted recovery of all survivors. Al-

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though alkali treatment and lysozyme increased recovery of survivors on nonselective and selective media, the percentage of detected survivors that were sensitive to the selective agents was unchanged (Table 1). This indicated that alkali treatment and germination by lysozyme did not introduce an artifact, and that UHT treatment damaged both the germination and outgrowth systems of most spores. Once germinated by lysozyme these spores also underwent repair and regained resistance to the selective agents (Fig. 3). Repair was complete within 3 h, as shown by equivalent CFU on the selective and nonselective media. Despite the lower incubation temperature, repair of alkali-treated spores was more rapid than repair of non-alkali-treated spores (Fig. 1), possibly because of more rapid germination.

Repair of injured spores also occurred in meat broth and milk. After a UHT treatment (105 C for 8.5 min), the number of surviving spores in TSN broth as indicated by the most-probable-number index was <0.3/ml. In BASE broth, meat broth, and milk the most-probable-number indexes were 2,400/ml, 640/ml, and 150/ml, respectively. The 95% confidence limits for BASE broth and foods overlapped each other, yet none of these overlapped the 95% confidence limit for TSN broth. The lack of growth in TSN broth was indicative of injury, whereas growth in BASE broth demonstrated repair and growth of injured spores. Growth in meat broth and milk indicated that these foods could support repair, although not as well as BASE broth.

The data show that nearly all C. perfringens spores that survived UHT treatment were more sensitive to polymyxin and neomycin. These
TABLE 1. Effect of alkali treatment on sensitivity of UHT-treated (105 C for 5 min) spores to selective agents of TSN

| Medium          | Non-alkali treated\(^a\) | Alkali-treated\(^b\) |
|-----------------|--------------------------|---------------------|
|                 | CFU/ml                   | Recovery (%)        | CFU/ml           | Recovery (%)    |
| BASE            | 1.7 × 10^6               | 100                 | <3.0 × 10^6      | 0                |
| BASE plus lysozyme | 2.5 × 10^6               | 150                 | 6.9 × 10^7       | 100              |
| TSN             | 1.7 × 10^6               | 0.1                 | 7.0 × 10^5       | 0.1\(^d\)       |
| BASE plus polymyxin | 1.2 × 10^6               | 0.7                 | 5.0 × 10^6       | 0.7\(^d\)       |
| BASE plus neomycin | 3.3 × 10^4               | 0.2                 | 1.4 × 10^5       | 0.2\(^d\)       |

\(^a\) Recovery on BASE was taken as 100%.
\(^b\) Recovery on BASE plus lysozyme was taken as 100%.
\(^c\) All alkali-treated spores require lysozyme for germination.
\(^d\) Media contained lysozyme.

injured spores underwent repair during outgrowth in BASE broth and foods. The site of injury and specific requirements for repair are presently under investigation.

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