Ultrahigh-Temperature Activation of a Low-Temperature Bacillus subtilis Spore Germination System\(^1,2\)

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Exposure of Bacillus subtilis A spores to 115 to 125°C for several seconds activated a low-temperature germination system that remained dormant after a heat treatment that activated the normal L-alanine- and glucose-stimulated germination systems. The low-temperature germination system was characterized by an optimum temperature lower than that of the L-alanine or glucose germination systems (30°C versus 45°C), germination in the absence of exogenous germination stimulants, and the capacity for heat-induced deactivation and subsequent reactivation. The rates of activation at 115 to 125°C were exponential and were not influenced by a previous heat treatment that activated the L-alanine- or glucose-stimulated germination systems. Although activation of the low-temperature germination system was accompanied by suppression of L-alanine-stimulated germination, it did not appear to be a modification of the L-alanine germination system.

Spores that survive lethal heat treatments frequently are recovered best at temperatures below the optimum growth temperature of nonheated or mildly heated spores (3–5, 13, 15). Recovery of lethally heated Clostridium botulinum spores at 20 to 27°C was greater than at 31 to 41°C (13, 15, 16). Spores of putrefactive anaerobe (PA) 3679 NCA strain behaved similarly (16). The incubation temperatures that yielded maximum colony counts for Bacillus stearothermophilus spores decreased from 50 to 65°C to 45 to 50°C after the spores were heated for 10 to 70 min at 115°C (3). The apparent number of B. subtilis A spores that survived ultrahigh temperature treatments was greater when survivors were incubated at 32°C instead of 45°C; whereas, plate counts of nonheated or mildly heated spores were greater at 45°C. When the plating medium was supplemented with CaCl\(_2\) and Na\(_2\) dipicolinate, the plate counts for heated or nonheated spores were the same at 32 and 45°C (4, 5).

Treatment of milk at 90.6 to 96.1°C produced a product with good shelf life; however, milk processed at 98.9 to 110.0°C showed a rapid loss of keeping quality (14). The apparent number of B. subtilis A spores that survived ultrahigh temperature treatment increased up to 10-fold during storage in skim milk at 3°C (5). Adams and Busta (1) observed increased germination of B. subtilis A spores in defined media at temperatures below 35°C after the spores were heated at 121°C for 6 sec.

The detection of surviving spores requires activation, germination, outgrowth, and vegetative growth of the survivors. In many of the above reports, it was not known which of these stages was affected by the lethal treatment that resulted in a lower optimum recovery temperature. The work of Edwards et al. (4, 5) and Adams and Busta (1) suggested that the affected stage was germination. The objectives of this investigation were to determine (i) if exposure to ultrahigh temperatures increased the germination of B. subtilis A spores at temperatures below the optimum germination temperature of unheated spores and (ii) if such an increase was a modification of the standard L-alanine or glucose germination systems or activation of a new germination system. A preliminary report of these findings has been made (D. M. Adams, F. F. Busta, and M. Jungbluth, Bacteriol. Proc., p. 63, 1971).
MATERIALS AND METHODS

Test organism. B. subtilis strain A was originally obtained from Z. John Ordal (University of Illinois, Urbana). Stock cultures were grown on fortified nutrient agar slants at 44 C for 24 hr and stored at 2 C. Growth and preparation of spore suspensions were described by Busta and Adams (2).

Media. The plating medium was fortified nutrient agar supplemented with 44 mM CaCl2 plus 40 mM sodium dipicolinate (CNA) (K & K Laboratories, Inc., Plainview, N.Y.). The composition and preparation of CNA were described by Edwards et al. (4). Each constituent of the germination media was prepared as a 10-fold concentrate, autoclaved at 121 C for 15 min, and when used was diluted with other germination medium constituents, deionized water, and spores.

Heat treatments. The primary or standard heat activation treatment for the L-alanine- and glucose-stimulated germination systems was 90 C for 60 min. The spores were suspended at a concentration of 109/ml in 25 mM sodium phosphate buffer, pH 7.

The secondary heat treatments were at 115 to 125 C and were preceded by a primary treatment. The spores were contained in sealed capillary tubes as described by Busta and Adams (2) except that the heating menstruum was 250 mM sodium phosphate buffer, pH 7, and the spore concentration was ca. 1010/ml.

Germination. Germination was measured as the reduction in optical density (OD) (expressed as a percentage of the initial OD) at 650 nm using a Turner model 330 spectrophotometer (G. K. Turner, Associates, Palo Alto, Calif.), loss of heat resistance (90 C for 30 min), or phase darkening (Zeiss Universal Microscope, Carl Zeiss, Oberkochen, West Germany).

RESULTS

Adams and Busta (1) observed that spores which had received primary (90 C for 60 min) and secondary (121 C for 6 sec) heat treatments germinated to a greater extent in 1 hr at temperatures below 40 C than did spores that had received only a primary heat treatment. The influence of incubation temperature on the germination in L-alanine of primary treated and secondary treated B. subtilis A spores is shown in Fig. 1. After secondary treatment, germination in L-alanine at 45 C was greatly reduced. Below 40 C the germination rate of secondary treated spores was lower than had been observed at 45 C for spores that received only a primary treatment. However, after 6 hr the extents of germination at temperatures below 40 C were greater with secondary treated spores than with primary treated spores. The optimum temperature for the germination of secondary treated spores in L-alanine was 30 C and reduced germination was observed at 45 C. At 30 C the extent of germiation of secondary treated spores equaled the extent of germination at 45 C of spores that received only a primary treatment. A similar experiment used glucose as the sole germination stimulant (Fig. 2). The secondary treatment did not reduce germination activity at 45 C in glucose. However, increased germination activity was observed below 35 C after 6 hr of germination. The apparent optimum temperature for germination in glucose by spores that received both primary and secondary treatments was 30 C. The influence of temperature from 10 to 30 C on the germination of secondary treated spores in glucose was similar to that observed when L-alanine was the sole germination stimulant (Fig. 1). Therefore, the low-temperature germination of secondary treated spores did not appear to be specific for L-alanine or glucose.

The data in Fig. 3 show that, unlike the germination of primary treated spores at 45 C, the germination of secondary treated spores at 25 C did not require exogenous germination stimulants. At 45 C the rate and extent of germination of primary treated spores was increased by L-alanine, glucose, or both. However, secondary treated spores germinated
equally well at 25°C in optimum levels of L-alanine, glucose, or both, and similar germination occurred in a NaCl-Na phosphate mixture or deionized water. D-Alanine, a potent inhibitor of the L-alanine-stimulated germination of primary treated spores at 45°C, had no effect on low-temperature germination. Therefore, the germination system activated by the secondary treatment (121°C for 9 sec) was independent of exogenous germination stimulants and appeared to be unrelated to the inactivated L-alanine germination system.

Activation of the low-temperature germination system during secondary treatment is shown in Fig. 4. The degree of activation, measured as the extent of germination in 6 hr at 25°C, increased exponentially with longer exposures at 115, 121, and 125°C (Fig. 4A). Previous exposure to the primary treatment was not required and did not affect the rate of activation by the secondary treatment (Fig. 4B).

Activation of the low-temperature germination system by secondary heat treatments was reversible. The data in Fig. 5 show that after a primary treatment, no germination at 30°C was observed after 28 hr in a germination medium of NaCl and Na phosphate buffer, pH 7. A secondary treatment of 121°C for 9 sec with or without a previous primary treatment activated the low-temperature system, and greater than 90% germination occurred in 28 hr at 30°C. When the secondary treatment was followed by a primary treatment, no germination occurred. The reduction in germination activity was heat-induced dormancy and not death.
because the low-temperature germination system was reactivated by another secondary treatment. After the reactivation, the rate of germination was lower than after the activation treatment, but germination at 24 to 28 hr was complete after correction for the reduced number of survivors.

Up to eight washings in deionized water did not reduce the germination of secondary treated spores at 30 C (Table 1). Therefore, any endogenous germination stimulant formed or released during secondary treatment was not freely diffusible. The medium in which secondary treated spores had germinated at 25 C did not stimulate low-temperature germination of spores that received only a primary treatment (Table 2).

After secondary treatment, germination occurred at as low as 4 C in a NaCl-Na phosphate mixture (Fig. 6). Germination was measured as phase darkening and loss of heat resistance. Greater than 90% germination occurred in less than 10 days, but after germination some of the spores died. The number of viable spores dropped during incubation at 4 C but the percentage of nonre refractile spores increased. Therefore, loss of viability occurred after and not before germination. The spores did not die immediately upon germination. This was indicated by the increase in the percentage of spores that were heat sensitive calculated as a fraction of the total viable population on the day of sampling.

**DISCUSSION**

Greater recovery of survivors at incubation temperatures below the optimum for the recovery of nonheated or heat-activated spores has been reported for spores of *Bacillus* and *Clostridium* spp. (3-6, 13, 15, 16). The change in the optimum recovery temperature has generally been attributed to a change in the optimum growth temperature, but the affected stage in the development of visible growth from the surviving spore has not been elucidated.

The data presented in this study show that exposure of *B. subtilis* A spores to secondary treatments activated a germination system that was inactive in spores that received only a primary treatment (90 C for 60 min). Low-temperature germination was verified as true

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**Table 1. Effect of washing on the germination of Bacillus subtilis A spores that had received a primary treatment or a primary and secondary treatment**

| No. of washings* | Primary treatment only | Primary and secondary treatment |
|------------------|------------------------|---------------------------------|
| 0                | 43.8a                  | 45.7a                           |
| 2                | 39.1                   | 48.7                            |
| 4                | 39.5                   | 48.4                            |
| 8                | 40.0                   | 49.1                            |

*Repeated centrifugation in 3 volumes of cold deionized water at 12,100 × g.

*Primary treatment, 90 C for 60 min; no secondary treatment; germination mixture: 10 mM L-alanine, 140 mM NaCl, 27 mM sodium phosphate buffer (pH 7), at 45 C for 1 hr.

*Primary treatment, 90 C for 60 min; secondary treatment, 121 C for 9 sec; germination mixture: deionized water (pH 7), at 30 C for 5 hr.

*Germination is measured in percent reduction in optical density.

**Table 2. Effect of exudates from secondary treated spores germinated at 25 C on the germination of primary treated spores at 25 C**

| Time (hr) | Percent reduction in optical density |
|-----------|-------------------------------------|
| 0.5       | 1.8b                                |
| 1.0       | 3.6                                 |
| 2.0       | 5.5                                 |
| 4.0       | 7.3                                 |
| 8.0       | 12.7                                |
| 21.0      | 17.5                                |

*Germination mixture: L-alanine, 10 mM; NaCl, 140 mM; pH 7 sodium phosphate buffer, 27 mM.

*Fresh germination medium.

*Spent germination medium of nonsecondary treated spores held at 25 C.

*Spent germination medium of secondary treated spores held at 25 C.
germination of primary treated spores occurred at subthreshold levels of L-alanine, and D-alanine completely prevented L-alanine-stimulated germination at 45°C. Secondary treated spores, however, were totally independent of an exogenous source of germination stimulants and were unaffected by D-alanine. The low-temperature germination which was activated by a secondary treatment was blocked by a subsequent primary treatment and was unaffected by a previous primary treatment. The standard L-alanine germination system was activated by a primary treatment and two consecutive primary treatments (i.e., 120 min at 90°C) had no deleterious effect (data not given). Finally, a previous primary treatment did not affect the rate of activation by the secondary treatment and may have increased the extent of activation after 4 sec at 121°C. The same primary treatment applied as a preheat greatly reduced the percentage of injured spores after a 4 sec exposure at 121°C (1).

Although there are other reports of low-temperature germination, no system resembled the one studied here. Halvorson, Wolf, and Srini-vasan (7) reported that B. cereus T spores germinated at -3 and -6°C in L-alanine or L-alanine plus adenosine. Wolf and Mahmoud (17) observed that spores of B. cereus var. thuringensis and B. subtilis germinated at 5°C in nutrient broth but not in salt solution. Germination of secondary treated spores at low temperatures in NaCl-phosphate buffer or water suggests that this germination system is unique.

The mechanism of low-temperature germination and its activation by secondary treatments is unknown. Dipicolinate released during lethal heat treatments may be involved. The optimum temperature for the germination of B. subtilis A spores in CaCl₂ plus Na₂ di- picolinate (DPA) was between 10 and 30°C (12). Ordal and co-workers (10, 11) reported that B. megaterium spores were activated by CaDPA for germination in water at low temperatures, and heating B. megaterium spores at 60°C in CaDPA prevented subsequent germination in CaDPA. Similarly, secondary treated spores germinated in water at low temperatures with an optimum of about 30°C. Low-temperature germination was prevented by heating secondary treated spores at 90°C in buffer. Exudates of low-temperature germinated spores did not stimulate germination at 25°C by primary treated spores, and repeated washings did not prevent low-temperature germination, suggesting that any chemical

![Graph](image-url)
stimulants of low-temperature germination was endogenous. If this stimulant were DPA, heating secondary treated spores in buffer could produce an effect similar to heating in a CaDPA solution.

Edwards et al. (5) reported that B. subtilis A spores, injured by exposure to 113 to 131°C, underwent repair during storage at 3°C. Repair was measured as the regained ability to form a colony on a nutrient agar medium. This apparent repair may have been low-temperature germination. Both repair and low-temperature germination occurred at 3 to 4°C; the extent of repair and germination increased with increasing ultrahigh temperature treatments; and both were followed by a delayed loss of viability. Adams and Busta (1) suggested that the injury observed on complex media by Edwards et al. (5) involved the inactivation of the L-alanine-stimulated germination system of B. subtilis A spores. They observed no repair of this germination system when the injured spores were heated at 90°C before low-temperature storage. This heat treatment also prevented low-temperature germination of secondary treated spores. Low-temperature germination could bypass a heat-induced block in normal germination. Upon plating, any such spores with normal outgrowth and vegetative cell growth systems would appear to have undergone repair.

Activation of the low-temperature germination system concurrently with the inactivation of the L-alanine germination system (1) appears to explain the reduction in the temperature optimum for the recovery of ultrahigh temperature treated B. subtilis A spores (4, 5). This may also explain similar observations with heated B. stearothermophilus (3), C. botulinum (13–15), and PA 3679 (16) spores. Holmes, Nags, and Levinson (8), working with B. megaterium spores, also observed suppression of one germination system (L-alanine) during the heat activation of another (glucose) system. However, the newly activated system had a higher, not lower, temperature optimum than the suppressed system (9).

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