Identification of a Germination System Involved in the Heat Injury of *Bacillus Subtilis* Spores

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Received for publication 30 December 1971

*Bacillus subtilis* A spores were injured by exposure to heat treatments of 110 to 132 C. Injury was demonstrated by the inability to form colonies on fortified nutrient agar (FNA) unless the medium was supplemented with CaCl₂ and Na₂ dipicolinate (CNA). A preliminary heat treatment fully heat-activated the spores, was not lethal, and did not prevent injury by subsequent secondary heat treatment. Exposure of heat-activated spores to 122 C reduced germination in FNA. The primary germination agents in FNA were identified, and a defined germination medium of glucose, NaCl, L-alanine, and sodium phosphate (GNAP) was developed. Germination of heat-activated spores in GNAP was equivalent to germination in FNA. Injury measured by colony formation on FNA and CNA was correlated to injury measured by reduced germination in both FNA and GNAP. Inactivation of the FNA and GNAP germination systems by secondary treatment exhibited similar kinetics. Therefore, injury expressed as the inability to form colonies on FNA involved alteration of the GNAP germination system.

The detection of surviving spores is required for thermal process evaluation, and the inability to accurately predict the survivors quantitatively has both economic and public health significance. Survivors of thermal processes may be injured and unable to grow under cultural conditions that are satisfactory for unheated spores. Little information is available as to whether germination, outgrowth, or vegetative cell growth is affected in heat-induced spore injury. Levinson and Hyatt (11) reported that for *Bacillus megaterium* at least 94% of the spores heated at 75 to 85 C retained germinability but were unable to form colonies due to damage to the cell-division process. Less than 6% were nonviable because of the lost ability to germinate. Campbell et al. (4) reported the isolation of germination mutants of *B. stearothermophilus* from spores heated at 121 C. Unlike unheated spores, the injured spores and their progeny were unable to form colonies on a minimal medium unless certain amino acids were added. Colony formation was the criterion of injury and survival; therefore, the authors could not distinguish between germination, outgrowth, or vegetative cell growth as the stage affected by the apparent mutations. Cassier and Sebald (5) observed improved recovery of heated *Clostridium perfringens* spores when egg yolk emulsion or lysozyme was added to the plating medium. Duncan et al. (6) recently reported that lysozyme germinated the injured *C. perfringens* spores whose normal germination system had been inactivated by heat treatment. Edwards et al. (7, 8) reported that *B. subtilis* A spores were injured by exposure to ultrahigh temperatures (UHT). The injured spores did not form colonies on a fortified nutrient agar unless the medium was supplemented with CaCl₂ and Na₂ dipicolinate. This suggested that *B. subtilis* A spore injury involved the germination systems.

The objectives of this investigation were to identify the germination system(s) active when *B. subtilis* A spores were enumerated in FNA and to determine whether this germination system(s) was involved in the UHT injury. Preliminary reports of these findings have been given previously (F. F. Busta, Bacteriol. Proc., p. 3, 1966; F. F. Busta, Bacteriol. Proc., p. 122, 1967).
MATERIALS AND METHODS

Test organism. B. subtilis strain A was originally obtained from Z. John Ordal (University of Illinois, Urbana). Stock cultures were maintained on fortified nutrient agar slants (7). The inoculated slants were incubated at 44 C for 24 hr and stored at 2 C.

Media. The plating media were fortified nutrient agar (FNA) and FNA supplemented with 44 mm CaCl₂ and 40 mm sodium dipicolinate (K & K Laboratories, Inc., Plainview, N.Y.) (CNA). The composition and preparation of the FNA and CNA were described by Edwards et al. (7). Constituents of the germination media were autoclaved separately at 121 C for 15 min and stored at room temperature.

Preparation of spore suspensions. Portions (0.5 ml) of a 16-hr nutrient broth (BBL) shake culture grown at 45 C were dispensed into 100 large petri dishes (150 by 22 mm) containing 100 ml of FNA, and were spread over the entire agar surface. The plates were incubated upright for 24 hr at 44 C in a water-jacketed, natural convection incubator, inverted and incubated for an additional 24 hr, and then stored at 4 C for 18 to 24 hr. The growth was harvested with cold, sterile deionized water, and the spores were washed and purified by centrifugation according to the procedure of Edwards et al. (7). To obtain spore crops of less than 1% vegetative cells required 10 to 15 centrifugations and washings. The spore crops were stored at 2 C in deionized water.

Heat treatments. The heat activation or primary treatment was 90 C for 60 min. The spores were suspended at a concentration of 10⁷/ml in 25 mm Na phosphate buffer, pH 7, and were heated in a covered water bath.

The secondary or damaging heat treatments were at 110 to 132 C. The spores were suspended in 25 mm Na phosphate buffer, pH 7, at a concentration of ca. 10⁷/ml. When germination was measured as a reduction in optical density (OD), the suspension for both the primary and secondary treatments contained ca. 10⁸ spores/ml. This suspension was dispensed in 0.04- or 0.05-ml quantities into glass capillary tubes (Kimble 34507; 0.9 to 1.1 by 90 mm) using a syringe equipped with a repeating dispenser (Hamilton Co., Whittier, Calif.), and the capillaries were sealed in a gas flame. The capillaries were placed in a wire mesh basket and immersed in a constant-temperature oil bath (Colora Ultra-Thermostat, Germany). The heat treatment was terminated by plunging the capillaries into an ice bath. Exposure time was measured with an electric timer (Precision Scientific, Chicago, Ill., model 69230) calibrated in 0.1-sec units. The temperature change during come-up was measured with a copper-constantan thermocouple implanted in a sealed, water-filled capillary tube and was recorded on a Speedomax G recorder (Leeds & Northrup Co., Philadelphia, Pa.). The heat contribution during come-up was calculated by the method of Halvorson (10) assuming a zₜ of 10 C. Six seconds were required for temperature equilibration, and the contribution during come-up was equivalent to 3 sec at bath temperature.

When germination was measured by change in OD, the capillaries containing the heated spores were washed, both ends were broken off, and the contents were blown into a small test tube. The spores were diluted 1 to 10 with deionized-distilled water and used in the germination experiment. When injury was measured by colony counts or germination was measured by loss of heat resistance, two capillaries that each contained 0.05 ml of heated spore suspension were washed, rinsed in sterile deionized water, and crushed in a 99-ml phosphate buffer dilution blank (3). The samples were then dispensed in germination media or were further diluted as described in Standard Methods for the Examination of Dairy Products (3), plated in triplicate on FNA or CNA, and incubated for 18 to 24 hr at 44 C.

Germination. Germination was measured as a loss of heat resistance or as a reduction in OD at 625 nm. When the germination medium contained agar, the agar was melted, cooled to 43 C, and added to the germination mixture. Germination was initiated by the addition of spores to the germination mixture after the system had been allowed to equilibrate to germination temperature. When measured as loss of heat resistance, germination was terminated by exposing the entire mixture of substrate and spores to 90 C for 15 min and then diluting the mixture immediately before colony count with CNA.

RESULTS AND DISCUSSION

Effects of multiple heat treatments on the apparent viability of B. subtilis A spores measured on FNA or CNA are shown in Fig. 1. Treated spores received a primary or heat-activation treatment (90 C for 60 min) prior to exposure at 121 C. Germination in FNA required heat activation and the primary treatment fully activated the spores. The number of primary treated spores enumerated on FNA and CNA was approximately equal to the direct microscopic count (data not presented). The plate counts of untreated spores on CNA were similar to those of primary treated spores plated on FNA. This indicated that the Ca dipicolinate (CaDPA) in CNA medium germi-
nated the spores directly and without previous heat activation. Riemann (12) also observed the germination of B. subtilis A spores by CaDPA. The primary treatment was not lethal as shown by equivalent plate counts on CNA before and after primary treatment.

The injury induced by the secondary treatment was demonstrated by the difference between the apparent number of survivors enumerated on FNA and CNA. After a secondary treatment, a spore was considered dead if it was unable to form a colony on CNA, and a viable spore unable to form a colony on FNA was considered injured. The presence of CaDPA in CNA overcame the damage of injured spores but had no apparent effect on the additional cellular damage in dead spores. This was also observed by Edwards et al. (7, 8) for spores heated in skim milk. The action of CaDPA as a germination agent for B. subtilis A spores and the requirement for outgrowth and vegetative cell growth for colony formation by injured spores on CNA strongly indicated that the site of injury was the spore germination system.

Heat-activated spores that received a primary treatment appeared to be more resistant to the damaging effects of the secondary treatment. Similar observations were made by Carawan (M.S. thesis, North Carolina State University, Raleigh, 1970) who reported that a sublethal heat treatment protected B. subtilis A spores against thermal inactivation by subsequent UHT treatments. However, up to 90% of the primary treated spores surviving exposure at 121 C were injured. Therefore, the primary or heat activation treatment required for germination in FNA did not interfere with a study of the influence of secondary treatments on spore germination.

Data on germination in FNA and CNA by primary treated and primary plus secondary (121 C for 6 sec) treated spores are shown in Fig. 2 and 3, respectively. Germination was measured as the loss of resistance to heat treatment at 90 C which also served to melt the agar when germination was carried out at 30 or 37 C. The controls indicate germination in 2% agar. With primary treatment only (Fig. 2), germination in FNA and CNA at 43 C were similar and greater than 90% germination occurred in 2 to 4 hr. Germination in FNA increased with increasing temperatures. The extent of germination in CNA, however, was much less influenced by temperature. This indicated that germination by CaDPA was either unaffected by temperature or increased with decreasing temperature, and thus compensated for the reduced germination in the FNA base of the CNA medium. Riemann (12) reported that the optimum temperature for CaDPA germination of B. subtilis A spores was between 10 and 30 C, and germination at 45 C was greatly reduced because of crystallization of the CaDPA.

After secondary treatment (Fig. 3), germination in FNA at 43 C was reduced by ca. 50%. A direct relationship between germination temperature and the extent of germination in FNA also existed for secondary treated spores. In CNA, however, secondary treated spores exhibited an inverse relationship between germination temperature and the extent of germination. This reflects reduced germination of the spores by CaDPA above the 30 C. The injured spores were unable to germinate at 43 C in the FNA base of the CNA medium. About 80% of the spores capable of germination in CNA after primary treatment germinated in CNA at 30 C after primary plus secondary treatment. Complete germination of injured spores for plate counts at 43 C when CNA was used to enumerate survivors (Fig. 1) apparently occurred because of the lower temperatures during and after solidification of the CNA medium in the pour-plate technique. It was apparent, therefore, that the inability of injured B. subtilis A spores to form colonies on FNA was due to the inability of these spores to germinate unless CaDPA was added to the medium.

The involvement of germination in heat injury necessitated identification of the germination system(s) that responded to FNA constituents and was suppressed by secondary heat treatment. This required evaluation of the FNA constituents, alone and in combination, for germination of uninjured and injured spores (Table 1). Germination in FNA after primary treatment or primary plus secondary treatment was similar to that shown for 90 min in Fig. 2 and 3. Deletion of minerals, Gelysate, or NaCl had no effect on the germination of spores that had received only a primary treatment. Removal of beef extract slightly reduced germination, and deletion of glucose severely retarded germination. Minerals, Gelysate, and especially glucose appeared to be important for the germination of spores that had also been subjected to a secondary treatment, although only a fraction of the survivors were able to germinate in the total or complex medium. Glucose was the only individual FNA constituent that by itself stimulated germination of heat-activated (primary treated) spores. Other FNA constituents in combination with
glucose increased germination, and germination in glucose, NaCl, and beef extract approached the germination observed in FNA for primary treated and primary plus secondary treated spores.

Amino acid analysis (Beckman amino acid analyzer) of FNA indicated the presence of 11 amino acids at the following concentrations: arginine, 1.65 mm; lysine, 0.76 mm; alanine, 0.62 mm; leucine, 0.49 mm; glycine, 0.44 mm; methionine, 0.33 mm; phenylalanine, 0.32 mm; tyrosine, 0.24 mm; isoleucine, 0.22 mm; serine, 0.19 mm; glutamic acid, 0.02 mm. For germination of primary treated and primary plus secondary treated spores in defined media, the media constituents were at the concentrations found in FNA; glucose, 0.55 mm; NaCl, 140 mm; Na phosphate, 2 mm; amino acids as given above. In glucose and the full complement of amino acids plus NaCl and phosphate, germination approached that observed in FNA. Maximal germination in L-alanine required the presence of other FNA constituents, especially glucose and NaCl. The defined germination medium (GNAP) used in future experiments therefore contained glucose, 0.55 mm; NaCl, 140 mm; L-alanine, 0.62 mm; and Na Phosphate, 2 mm. After secondary treatment the extent of germination was reduced, as was the influence of the germination medium composition.

Data describing the germination of *B. subtilis* spores in defined media are presented in Table 1.

**Table 1. Germination of primary treated and primary plus secondary treated *Bacillus subtilis* A spores in defined media**

| Germination medium | Primary treatment | Primary + secondary treatment |
|--------------------|-------------------|-----------------------------|
| FNA*              | 100               | 100                         |
| FNA – minerals     | 95.5              | 27                          |
| FNA – Ge lysate    | 95                | 33                          |
| FNA – NaCl         | 99                | 103                         |
| FNA – beef extract | 80                | 70                          |
| FNA – glucose      | 35                | 0                           |
| Agar (A)           | 5                 | 0                           |
| Minerals (M)       | 0                 | 40                          |
| Gelysate (Ge)      | 0                 | 0                           |
| NaCl (N)           | 0                 | 0                           |
| Beef extract (B)   | 0                 | 0                           |
| Glucose (G)        | 31                | 23                          |
| G + M              | 30                | 67                          |
| G + Ge             | 41                | 63                          |
| G + N              | 57                | 43                          |
| G + B              | 56                | 70                          |
| G + N + M          | 52                | 30                          |
| G + N + Ge         | 76                | 43                          |
| G + N + B          | 90                | 60                          |
| G + A              | 33                | 40                          |
| N + G + A          | 56                | 87                          |
| Phosphate (P) + N + G + A | 66 | 97 |
| Amino acids + P + N + G + A | 87 | 107 |
| Amino acids – L-alanine + P + N + G + A | 79 | 133 |
| L-Alanine + A      | 17                | 110                         |
| L-Alanine + P + A  | 19                | 83                          |
| L-Alanine + N + A  | 55                | 110                         |
| L-Alanine + G + A  | 86                | 100                         |
| L-Alanine + P + G + A | 101            | 77                          |
| L-Alanine + P + N + G + A | 112 | 100 |

* Measured after 90 min at 43 C as the loss of heat resistance.
* Measured after 90 min at 43 C as the loss of heat resistance.
* 121 C/6 sec.
* FNA, fortified nutrient agar.
A spores in GNAP after primary treatment and primary plus secondary treatment are shown in Fig. 4. The primary treatment increased germination in the defined medium by about threefold as had also been observed for colony formation in FNA (Fig. 1). After a primary and secondary treatment, germination in GNAP was reduced by 55 to 60% from that observed for primary treated spores. This agreed well with the 57% reduction in germination after 90 min in FNA (Fig. 2 and 3).

A comparison of the effects of secondary treatment on the germination of heat-activated spores in FNA and GNAP is shown in Table 2. After primary treatment, the spores germinated to about the same extent in FNA and in GNAP. Injury induced by each secondary treatment and measured on FNA and CNA was accompanied by similar but reduced germination in both FNA and GNAP. After a primary treatment and a secondary treatment of 122 C for 6 sec, 82% of the spores retained viability as measured on CNA. Of these survivors, however, only 41% and 38% germinated in FNA and GNAP, respectively. These values correspond to reductions in germination activity (relative to the controls) of 52% and 59% for FNA and GNAP, respectively. These values are in agreement with others obtained when germination was measured as a loss of heat resistance (Fig. 2, 3) or as a reduction in OD (Fig. 4). Injury measured as reduced germination was greater than injury measured by colony formation on FNA and CNA. This may have been due to slow germination in FNA via a germination system not requiring germination stimulants and activated by the secondary treatment (2). This germination of injured spores would increase the plate counts on FNA thus reducing the apparent injury, especially since injury studied here does not appear to involve outgrowth or vegetative cell growth. The reductions in FNA- and GNAP-stimulated germination activity after exposure to secondary treatment had similar kinetics (Fig. 5). This confirmed that the GNAP medium included the germination agents active in FNA and verified the existence of the classical l-alanine-glucose germination system(s) operative in these spores (9). Also, a significant amount of the injury observed on FNA can be attributed to the effects of secondary heat treatments on the GNAP germination system(s). These findings on damage of a spore germination system are consistent with other findings reported by Adams and Busta (1). These alterations of this germination system(s) can be studied using the defined medium. Insight into the mechanism by which lethal heat treatments alter spore germination characteristics would improve understanding of spore germination and death. Such information should also permit establishment of recovery conditions more suitable for the detection of injured spores.

![Graph](Fig. 4. The influence of primary (P) and secondary (S) heat treatments on the germination of Bacillus subtilis A spores in the defined medium. Open circles are control spores that received no heat treatment. P, 90 C for 60 min; S, 122 C for 6 sec.)

| Temp (C) | Time (sec) | Surviv-al% | Injury% | Germinationc% |
|---------|------------|------------|---------|---------------|
|         |            |            |         | FNA | GNAP |
| Heat-activated control | 500 | 100 | 0 | 85 | 93 |
| 110 | 30 | 45 | 15 | 55 | 62 |
| 115 | 60 | 20 | 31 | 42 | 31 |
| 122 | 6 | 82 | 21 | 41 | 38 |
| 127 | 9 | 50 | 36 | 26 | 0 |
| 132 | 2 | 76 | 25 | 36 | 42 |
|       | 4 | 39 | 36 | 3 | 7 |
|       | 0.5 | 89 | 7 | 49 | 53 |

* Percent survivors determined by colony counts on fortified nutrient agar supplemented with CaCl2 and Na2 dipicolinate (CNA).
* Percentage of the survivors unable to form colonies on fortified nutrient agar (FNA).
* Germination measured as the percentage of the survivors losing heat resistance during 90 min at 43 C in FNA or a defined germination medium of glucose, NaCl, l-alanine, and sodium phosphate (GNAP).
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