Germination-Initiated Spores of Bacillus brevis Nagano Retain Their Resistance Properties

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Initiated spores and vegetative cells of the gramicidin S-producing Bacillus brevis Nagano were compared with respect to their resistance to various forms of stress (osmotic shock-starvation, exposure to ethanol, sonic oscillation, and heat). The resistance of initiated spores to all of these stress situations was considerably greater than that of vegetative cells and approached that of dormant spores. The period during which the initiated spores remained resistant to heat was extended by addition of gramicidin S. The antibiotic may therefore be of survival value to the species in nature by slowing down the development of initiated spores in the outgrowth phase of germination, thereby extending the period during which the cells are resistant to environmental stress.

By the process of germination, a dormant Bacillus spore becomes a vegetative cell. This process is subdivided into four stages: activation, triggering, initiation, and outgrowth (20). Activation is a fast reaction which does not involve any degradative process and which increases the percentage of spores capable of germination. Activated spores do not, however, continue germination unless exposed to a germinant. Different low-molecular-weight compounds can act as germinants for spores of different species. For Bacillus brevis, L-alanine can serve as germinant (15). During the next phase, initiation, the spore loses 30% of its dry weight in a degradative process (1). Breakdown of protein (24–26), cortex (9), and lipid (20) occurs during initiation. As a result of excretion of dry matter, initiating spores show a drop in refractility and light absorption (17, 18). During initiation, spores of B. subtilis, B. cereus, and B. megaterium lose heat resistance (4, 8, 11, 17). During outgrowth, RNA synthesis occurs, followed by protein synthesis, and finally by DNA synthesis. The outgrowing spore becomes swollen and a vegetative cell begins to emerge. The spore coat is shed by the vegetative cell when it begins to divide.

Although many aspects of the biochemistry of sporulation and germination in bacilli have been investigated, one aspect is still not fully understood, i.e., the involvement of peptide antibiotics in differentiation. Because peptide antibiotics are produced during the early stages of sporulation, they have been implicated as regulators of spore formation (12–23). However, the existence of mutants that are unable to synthesize peptide antibiotics but sporulate normally presents strong evidence that antibiotics are not obligatory for sporulation (3, 10, 16, 30). Use of these abant and sp mutants has led to the discovery that the stage sensitive to the inhibitory effect of peptide antibiotics on their producing strain is that of outgrowth of germinating spores (5, 12, 13, 15). The inhibition of the outgrowth stage by endogenous antibiotic in antibiotic-producing strains might be viewed as a protective mechanism to prevent spores from entering the vegetative state in overcrowded, nutritionally deficient, or toxic environments (6, 28), or at least to give them more time to be transported to a less hostile environment.

We were however, unable to understand why it would be useful for B. brevis to be held in the state of outgrowth if outgrowing spores were indeed sensitive to heat and other environmental factors. We decided to see whether B. brevis behaves like other bacilli in the sensitivity of outgrowing spores to physical factors. This study therefore focuses on the resistance properties of initiated spores of B. brevis Nagano compared with those of dormant spores and vegetative cells. Surprisingly, we found initiated spores to be resistant to heat and other forms of stress.

MATERIALS AND METHODS

Strain. B. brevis Nagano, a producer of gramicidin S (15), was obtained from J. M. Piret, Massachusetts Institute of Technology.

Media. ALD salts contained the following components (in g/liter) in a 10-fold concentrated solution (2): K2HPO4, 30; KH2PO4, 10; NH4Cl, 0.005; NH4NO3, 1; Na2SO4, 1; MgSO4·7H2O, 0.1; MnSO4·H2O, 0.01; FeSO4·7H2O, 0.01; and CaCl2, 0.005 (pH 6.8 to 7.0). Salts I (100-fold concentrate) contained (in g/liter) CaCl2·2H2O, 10.3; MnCl2·4H2O, 1; and MgCl2·6H2O, 20.3. Salts II (100-fold concentrate) contained (in g/liter) FeCl3, 0.027 in 0.1 N HCl. NBS medium contained nutrient broth (Difco Laboratories) solids, 8 g/liter; and salts I and II, 10 ml/liter of each. Nutrient agar contained (in g/liter) nutrient broth solids, 8; and agar, 15.

Spore preparation, storage, and counts. Overnight seed cultures of B. brevis Nagano were prepared by inoculating cells or spores into 40 ml of NBS medium in 250-ml baffled shake flasks. The seed cultures were grown at 37°C on the shaker at 220 rpm until they reached the logarithmic growth stage. Baffled Fernbach flasks (2,800 ml) containing 300 ml of broth were inoculated with 7.5 ml of seed culture, and the cultures were incubated (37°C, 220 rpm) until sporulation was complete. The spores were harvested by centrifugation, washed four times in double-distilled water, suspended in water, transferred in 2-ml aliquots into small vials, and stored at −20°C. These suspensions contained 10⁸ to 10⁹ CFU per ml.

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Cell measurements. A Klett-Summerson colorimeter was used to measure cell density. Readings were taken in the linear range (below 150 Klett units) by using a red filter (no. 66).

Plate counts were done after diluting spore or cell populations in ALD salts. Plating was done in quadruplicate on nutrient agar plates. Since the experimental organism is motile and capable of spreading, the plates used for counts were predried at 37°C for 24 h after preparation to ensure the formation of distinct, countable colonies. The incubation temperature was 37°C.

Resistance properties of dormant spores, initiated spores, and vegetative cells. Samples (5 ml) of dormant spores, initiated spores, and vegetative cells (the last two were collected at 1 and 5 h, respectively) were centrifuged, washed four times with double-distilled water, and then suspended in 5 ml of double-distilled water or 70% ethanol.

Controls were incubated statically at room temperature and viable counts were taken at 0, 18, and 36 h. For sonic oscillation experiments, a 1-ml sample was placed in a plastic microcentrifuge tube and treated for 40 s (Bronson Sonifier-Cell Disruptor 200, output setting 4, 50% pulse, standard tapered microtip). Viable counts were taken before and after sonic oscillation to determine the percentage of cells killed by the treatment. Heat resistance as a function of temperature and time was measured by using watertight Hungate tubes. Tuberculin syringes (Becton-Dickinson and Co.) were used to inject 0.1 ml of each sample into duplicate Hungate tubes containing 10 ml of distilled water. The tubes were fully immersed in a water bath at 80°C for 0, 60, 120, and 180 min. The contents were diluted, plated, and counted.

Germination. Germination experiments were conducted with unbaffled 300-ml sidearm flasks containing 19 ml of NBS and 1 ml of inoculum (spores heat-activated for 30 min at 80°C). When gramicidin S was added to the germination

FIG. 1. Effect of exposure of dormant spores, initiated spores, and vegetative cells to a temperature of 80°C.

FIG. 2. Effect of exposure of dormant spores (open bars), initiated spores (hatched bars), and vegetative cells (solid bars) to three types of stress. Sonic oscillation (A) was done for 40 s. Exposure to water (B) and to 70% ethanol (C) was done for 36 h.
medium (0.2 ml of gramicidin S solution [1 mg/ml] in 50% ethanol), the control contained 0.2 ml of 50% ethanol. The ethanol (final concentration, 0.5%) had no effect on the germination kinetics. Germination was followed (i) turbidimetrically by absorbance measurements, (ii) by phase microscopy, and (iii) by measuring heat resistance at 80°C for 1 h.

RESULTS

Germination. In a typical germination experiment, absorbance decreases during initiation, remains fairly constant during outgrowth, and increases at the onset of vegetative growth. Since spores prepared in NBS medium possess only a low concentration of gramicidin S (15), the outgrowth period only lasts for ca. 1 h. However, upon addition of exogenous (10 μg/ml) gramicidin S, outgrowth is extended for 5 to 7 h.

Under phase microscopy, dormant spores appeared as phase-bright structures, initiated spore populations were virtually 100% phase-dark, and outgrowing spores were dark and swollen. Vegetative cells were motile, dark rods, considerably longer than the spores.

Heat resistance. Vegetative cells suffered a >99% loss in viability during 1 h of heating at 80°C, as expected. However, we were surprised to observe that initiated spores were very stable when exposed to this treatment.

Since spores of other bacilli are known to lose their heat resistance upon initiation of germination, a second experiment was done with a modified protocol to obtain equivalent populations of the three types of cells at zero time, to heat the cells for a longer time (up to 180 min), and to count the cells more accurately by including more plates for each time point. The results in Fig. 1 show again that initiated spores are considerably more resistant than vegetative cells.

Resistance to osmotic shock-starvation. Exposure to water for up to 36 h had no effect on survival of dormant spores and initiated spores (Fig. 2). On the other hand, 98% of the vegetative cells were killed, and these were already dead by 18 h of exposure to water (18-h data not shown).

Ethanol resistance. The results presented in Fig. 2 show that dormant spores and initiated spores are resistant to 70% ethanol, whereas vegetative cells are sensitive to this stress. Vegetative cells showed a drop in viability of 96% after only 18 h of exposure (data not shown).

Resistance to sonic oscillation. Spores and initiated spores were found to be resistant to sonic oscillation when compared with vegetative cells (Fig. 2). Vegetative cells showed a 98% loss in viability.

Effect of added gramicidin S on loss of heat resistance during germination. As reported previously (15), addition of gramicidin S to spores does not affect germination initiation as measured by the rate of decrease in culture absorbance and loss of spore refractility; however, outgrowth is delayed for several hours. The gramicidin S-induced delay in outgrowth is accompanied by a corresponding delay in loss of heat resistance (Fig. 3). Thus, initiated spores in the presence of gramicidin S retain their heat resistance for ca. 6 h. Similarly, spores prepared in LBS medium (15) contain larger amounts of endogenous gramicidin S than spores in NBS medium and retain their heat resistance for longer periods (data not shown).

DISCUSSION

A major part of the microflora of the soil is composed of spore-forming microorganisms. The importance of the dor-

![FIG. 3. Loss of heat resistance during germination of spores in the absence (A) and presence (B) of exogenous gramicidin S (10 μg/ml). Symbols: O, absorbance; ●, CFU.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
tant population of the soil and the concept that lack of nutrients keeps most of the population inactive most of the time have been pointed out (7, 27). Therefore, spores and their resistance properties are extremely important in the survival of microorganisms.

Understanding the physical and chemical characteristics which give dormant spores their resistance properties is an important step toward understanding how these resistances are developed during sporulation and lost during germination. Resistance describes the ability of the spore to go through the stages of germination and form a colony after being exposed to a particular treatment. Dormant bacterial spores are known to be more resistant than vegetative cells to chemicals, extremes of temperature, antibiotics, radiation, dyes, starvation, and several other environmental stresses (19, 29).

Studies of germination in B. megaterium, B. cereus, and B. subtilis have shown that initiated spores become sensitive to heat during the first few minutes of initiation (4, 8, 11, 17). B. brevis had not been studied in this regard, and our results surprisingly indicate that it behaves quite differently, in that the resistance of initiated spores approximates that of dormant spores. Since gramicidin S lengthens the outgrowth stage for many hours, it is now reasonable to postulate that gramicidin S production offers B. brevis a survival advantage in nature. A spore in nature may be activated by an elevated temperature in the presence of a germinant and initiate germination while still exposed to an unfriendly environment (e.g., too many competitor cells, presence of toxic compounds, or nutritional deficiency). By delaying development of the initiated spores to the sensitive vegetative state, the antibiotics could allow the species to test the environment for crowded, toxic, or nutrient-limited conditions before beginning vegetative growth. In the event of unsuitable conditions, the outgrowing spores might revert to dormant spores by microcycle sporulation (14, 31) or at least have more time to allow for transportation to a more friendly environment for vegetative growth. Whether or not B. brevis cells in nature produce enough gramicidin S to delay outgrowth remains to be established.

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LITERATURE CITED