Temperature Sensitivity of Sporulation in *Bacillus cereus*

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INTRODUCTION

Conditional heat-sensitive asporogenic mutants are useful for elucidating the functions involved in spore formation. Such mutants isolated from *Bacillus subtilis* exhibited heat sensitivity at either early (Leighton *et al.* 1972) or median stages (Leighton, 1973) of sporulation. Mutants of *Bacillus cereus* which sporulate at 28 °C but not at 37 °C have been described by Lundgren & Cooney (1962) and by Stelma & Sadoff (1973). We have observed that wild-type strains of *B. cereus* which grow and sporulate at 37 °C grew at 44 °C but failed to sporulate at that temperature. Comparative studies of morphological and physiological changes associated with sporulation were carried out at the permissive and at the restrictive temperature in a directed-sporulation system. Shift-up and shift-down experiments with *B. cereus* 569 in directed-sporulation culture show that some early functions involved in sporulation display heat sensitivity.

METHODS

**Organisms.** These were: *Bacillus cereus* NRRL569; *B. cereus* T; *B. cereus* ATCC6464; *B. cereus* ATCC7004; *B. subtilis* 168SB25 (try−, his−).

**Media.** Difco nutrient broth and PA medium (Thorne, 1968) were used for routine growth and sporulation. Directed-sporulation was carried out in PA medium modified to contain (g/l): Difco nutrient broth, 1·5; NaCl, 5; MgSO₄·7H₂O, 0·2; MnSO₄·H₂O, 0·05; CaCl₂·2H₂O, 0·3.

**Directed-sporulation conditions.** A vegetative inoculum was prepared from cultures grown in PA medium at 37 °C to a concentration of about 1 × 10⁸ colony-forming units/ml, equivalent to an extinction of 0·40 at 540 nm (Coleman Junior spectrophotometer). Bacteria were harvested by centrifuging, kept at 4 °C overnight and resuspended in sporulation medium (extinction 0·62 at 540 nm). The suspensions were shaken at 37 °C in flasks filled to 1/10th of their volume.

**Spore count.** Spores were counted after heat treatment at 80 °C for 30 min.

**Biochemical tests.** RNA synthesis in directed-sporulation culture was assayed by measuring the incorporation of [2-¹⁴C]uracil during 1 and 30 min pulses. Radioactivity was determined using a Packard ‘Tricarb’ liquid scintillation spectrometer. Aconitase activity was determined in extracts by the procedure of Racker (1950) using trisodium citrate as substrate. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Proteolytic activity of the crude supernatant solutions was determined according to the method of Millet (1970) with minor modifications. The substrate employed was 0·5 % Azoalbumin (Ε_{1%=35} = 35; Sigma) in 0·2 m-tris-HCl buffer pH 7·2.
RESULTS AND DISCUSSION

The yields of sporulation in *Bacillus cereus* 569 grown at 37 and 44 °C were found to be $2 \times 10^8$ spores/ml and $5 \times 10^5$ spores/ml, respectively. Sporulation of other strains, *B. cereus* T, *B. cereus* 6464 and *B. cereus* 7004, exhibited a similar heat sensitivity at the higher temperature. *B. subtilis* 168-8B, however, sporulated equally well both at 37 and 44 °C. The heat sensitivity of *B. cereus* was restricted to the sporulation phase since vegetative growth at 44 °C was hardly impaired. Shift-up experiments in the stationary phase showed that sporulation at 44 °C was inhibited independent of the vegetative growth temperature.

A directed-sporulation system was introduced to investigate the heat sensitivity of various stages of the sporulation process. Samples from cultures termed $t_0$ to $t_5$ were withdrawn at 1 h intervals and scored for heat-resistant spores. At 37 °C, forespores viewed under the phase microscope were prominent at $t_3$, followed by refractile spores at $t_4$. A high yield of heat-resistant spores was obtained at $t_5$. Incubation of directed-sporulation cultures at 44 °C, however, resulted in a low yield of spores; no morphological differentiation could be detected on microscopic examination. No change in the extinction of cultures was recorded at either temperature during period $t_0$ to $t_4$.

Shift-up experiments were carried out in this system. Samples transferred from 37 °C at 1 h intervals were incubated at 44 °C for up to 5 h and examined for spore yield. The sporulation temperature sensitivity was limited to stages $t_0$ to $t_2$, as shown by the low yield of spores in cultures shifted up at these stages (Fig. 1a). It appears that the heat-induced asporogenicity of *Bacillus cereus* is due to the inhibition of early events of sporulation and that the sporulating organisms become heat sensitive 2 h before their maturation into heat-resistant spores.

To determine whether sporulation capacity could be restored by transfer to the permissive temperature, directed-sporulation cultures at 44 °C were shifted down at 1 h intervals to 37 °C for an additional incubation period of 5 h. The heat-induced asporogenicity (Fig. 1b) could be reversed only if shift-down took place during the first 2 h at 44 °C ($t_0$ to $t_2$). The maximum spore yield in these cultures was $2 \times 10^9$/ml. In cultures shifted down at $t_3$, $t_4$ and...
Short communication

tₕ, spore yields were reduced to 2 x 10⁷/ml, 1.5 x 10⁶/ml and 2 x 10⁵/ml, respectively. Failure to sporulate after shift-down to 37 °C at tₕ to tₕ was not due to increased mortality at 44 °C. Hardly any change in the number of colony-forming units could be detected during the phase t₀ to t₂ at 44 °C. At tₕ, about 20 % of the initial colony-forming units could still be recovered. A temperature-sensitive irreversible event seems to occur early in sporulation, which does not preclude bacteria from re-entering their vegetative growth cycle at 37 °C since, when plated at 37 °C, they grew into spore-containing colonies.

Early stages of sporulation involve RNA precursor incorporation (Ramaley & Burden, 1970), a functional tricarboxylic acid cycle (Ramaley & Burden, 1970; Szulmajster, 1973), and derepression of the synthesis of proteolytic enzymes (Mandelstam & Waites, 1968; Szulmajster, 1973). At 37 °C, RNA precursor incorporation, enhanced activity of aconitase and a rise in the pH of the medium (from 5.9 to 7.1) were characteristic for stages t₀ and t₁; the appearance of a exoproteolytic activity followed at t₂. At 44 °C, [2-¹⁴C]uracil incorporation into both total RNA and the rapidly labelled fraction of RNA was smaller than that obtained at 37 °C. This finding is similar to that reported for a heat sensitive asporogenic mutant of Bacillus subtilis blocked at early stages of sporulation (Szulmajster, Bonamy & Laporte, 1970). An initial rise in aconitase specific activity at t₁ was followed by a decline in the activity at t₉, at both permissive and restrictive temperature. In contrast, an overproduction of this enzyme was reported for a heat-sensitive asporogenic mutant of B. subtilis under restrictive conditions (Leighton et al. 1972). It appears, therefore, that late vegetative functions are not altered in our system at 44 °C. Exoproteolytic activity characteristic of stages t₉ and tₐ at 37 °C was negligible at 44 °C. A similar observation has been reported for a conditional asporogenic mutant of B. subtilis (Leighton et al. 1972). The absence of exoproteolytic activity in B. cereus 569 at 44 °C was not due to inactivation of the enzyme at that temperature, since proteolytic activity in control supernatant solutions was not affected by the pre-incubation at 44 °C for 1 h.

Induction of sporulation is assumed to involve changes in the template specificity of RNA polymerase (Losick & Sonnenschein, 1969; Leighton et al. 1972; Szulmajster, 1973). In B. subtilis susceptibility to sporulation is limited to certain stages in the DNA replication cycle (Dawes, Kay & Mandelstam, 1971) and Szulmajster (1973) suggested that a specific location of DNA within the cell might be required for selective transcription of genes during sporulation. Our results with B. cereus 569 wild-type, suggest that during prolonged incubation at 44 °C the availability of the DNA template for transcription of sporulation genes might be irreversibly altered. We cannot however exclude the possibility that some vegetative functions, which are more essential for sporulation than vegetative growth, are damaged at the restrictive temperature.

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REFERENCES


Short communication


