CALCIUM UPTAKE BY *BACILLUS STEAROTHERMOPHILUS*: A REQUIREMENT FOR THERMOPHILIC GROWTH

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1. Introduction

Calcium accumulation by *Bacillus* species is known to occur during sporulation [1]. An active transport system for calcium has been found in *Bacillus megaterium* [2,3] and in *Bacillus subtilis* [4]. The former system appears to be active only during sporulation. Silver et al. [5] have even suggested that the physiologically significant calcium transport for bacteria in general occurs from the interior of the cells outward.

However, calcium, potassium, phosphate and an energy source were found to be necessary requirements for the survival of *Bacillus stearothermophilus* cells at elevated temperatures [6]. This finding suggests that an active transport of calcium ions into the cells is of fundamental importance for the heat stability of thermophilic bacteria.

We now present further evidence for the existence of a transport system for calcium during *vegetative* thermophilic growth of *B. stearothermophilus*. The uptake rate and the final amount of calcium associated with the cells increases with temperature. In our view, calcium stabilizes the cell functions against the action of heat. The general validity of other theories attempting to explain why thermophilic bacteria are able to grow at high temperatures has been discussed by Ljunger [6,7], and this subject has been reviewed recently [8].

2. Materials and methods

B. stearothermophilus, strain NCA 1503, is an obligately thermophilic organism. Under the experimental conditions described below, its minimum temperature is about 40°C and its maximum about 70°C. The sporulation frequency is very long during the stationary phase and any sporulating mutants are easily detected by way of altered colony morphology.

The organism was grown in tryptone starch broth (Bacto Tryptone, Difco, 10 g; soluble starch, Merck, 2 g; distilled water, 1000 ml). The concentration of calcium in this medium was approximately 0.05 mM, as measured by atomic absorption. Cultures in 50 ml liquid medium were grown in 250 ml Erlenmeyer flasks equipped with side tubes for nephelometric measurements. They were inoculated with 1 ml of an exponentially growing culture and incubated in thermostatically controlled waterbaths (± 0.1°C) with reciprocal shaking. The increase in turbidity of the cultures was registered with an EEL (Evans Electroselenium Limited) nephelometer. The nephelometer units (NU) are related to a given standard, and 100 NU corresponds to about 300 μg cellular dry weight per ml of a culture grown at 45°C (250 μg at 55°C). Dry weight of cells was determined on saline (0.85% w/v) – formalin (1% v/v) washed cells concentrated in distilled water and dried to constant weight at 105°C. Viable counts were made by diluting samples in tryptone-broth followed by spreading on tryptone agar (3%)-plates dried at 55°C. Colonies having developed after 20 h at 55°C were counted and viability expressed as colony forming units (CFU) per ml culture. Specific growth rates (k) were calculated from the doubling time T_D, during the early exponential phase, of the parameter studied ($k = \ln 2/T_D$).

Accumulation of ^{45}Ca was determined by rapid

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filtration of a culture sample on membrane filters using a sampling manifold (Millipore Corp.). Duplicate 0.2 ml samples from cultures containing 0.4 μCi ^{45}Ca (The Radiochemical Center, Amersham) per ml were rapidly removed and filtered. To obtain rapid filtration and to reduce nonspecific adsorption to filters, it was found necessary to use double filters. A 0.45 μm Millipore HA filter was overlaid with a 0.2 μm Nucleopore filter. Before filtration of the samples 2 ml of temperature adjusted sterile growth medium was passed through the filters. The Nucleopore filter was dried and placed in vials containing 14 ml of a toluene-based scintillation fluid (Omnifluor, New England Nuclear Corp.). Radioactivity on the filters was measured in a Beckman β-mate II scintillator at about 70% efficiency. Unfiltered 0.1 ml culture samples were dried on filters and counted to measure total radioactivity.

3. Results and discussion

First we investigated calcium accumulation at 55°C. Fig.1 shows data compiled from three experiments. The uptake of ^{45}Ca followed the general pattern of a growth curve. It was detectable about 0.5 h after inoculation and continued at an approximately constant rate for about 2 h. No additional uptake occured during the stationary phase contrary to the behaviour of sporulating *Bacillus* strains [3,4]. In contrast, both the viable counts and the amounts of cell associated calcium decreased after 3.5 h, whereas the nephelometer values remained constant. The maximum precentage of medium radioactivity found in the cells was about 10, which is considerably less than during sporulation. In the latter case all medium ^{45}Ca is ultimately taken up by the cells [2,4].

When a culture was shifted from 55°C to 20°C, well below the minimum growth temperature of

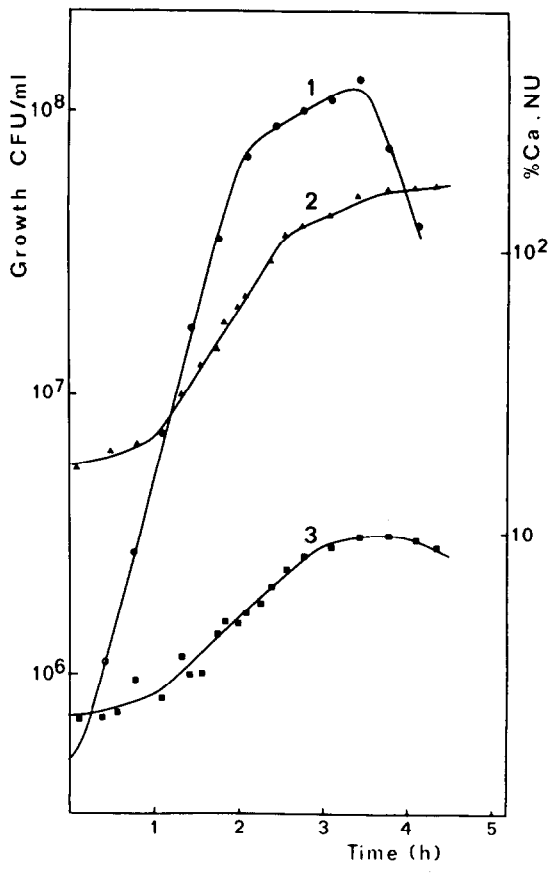

Fig.1. Calcium uptake by *Bacillus stearothermophilus* during vegetative growth at 55°C. Data compiled from 3 experiments. Curve 1: colony forming units (CFU) per ml. Curve 2: nephelometer units (NU) and Curve 3: percentage of medium calcium in cells calculated from uptake of ^{45}Ca.

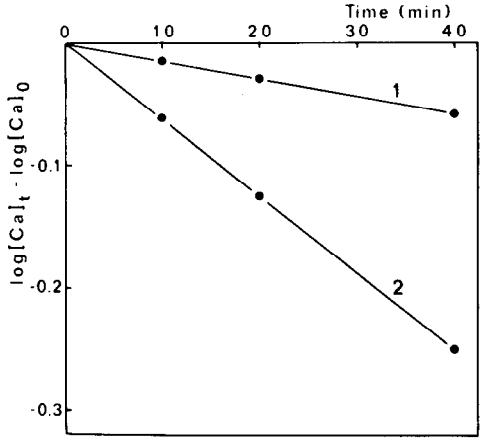

Fig.2. Effect of temperature shift and toluene on cell associated calcium. Two cultures took up ^{45}Ca at 55°C. At time 0 one was rapidly cooled to 20°C and maintained at this temperature (1). The other was kept at 55°C, but toluene (1% v/v) was added at time 0 (2).

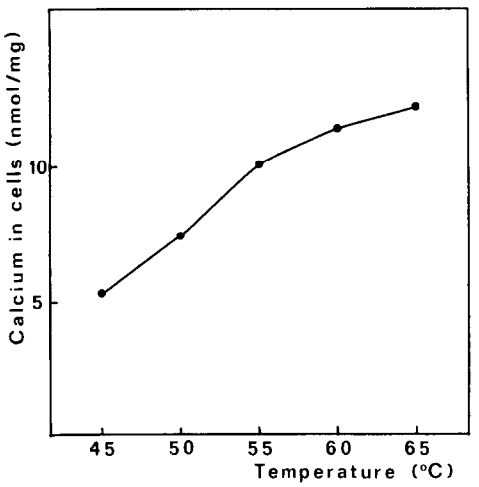

Fig.3. Effect of temperature on cellular calcium content. Averages estimated from uptake during the middle exponential phase.

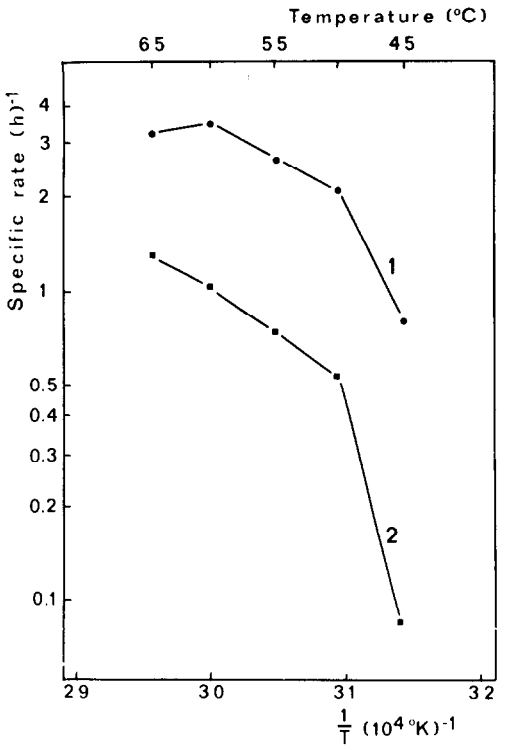

Fig.4. Effect of temperature on specific growth rate calculated from viable counts (1) and the specific calcium uptake rate (2).

B. stearothermophilus, growth stopped and a gradual release of accumulated calcium was evident as shown in fig.2. It was also found that toluene caused a rapid release of calcium at 55°C (fig.2: curve 2). In this case, however, the release was accompanied by a reduction in viable cells. After 40 min only about 0.5% of the cells had survived this treatment.

We concluded that calcium accumulation was not primarily due to a passive uptake, e.g. to adsorption, but was associated with the living state. An estimation of the cellular calcium content also showed that this value was highest during the middle exponential phase and lower during the stationary phase.

In order to compare the cellular calcium content at different temperatures, we extended the uptake studies in the range 45°C to 65°C. Fig.3 shows that more calcium is associated with the cells at higher temperatures. A rough calculation, assuming 4 μl cellular water per mg dry weight and that no intra- or extra-cellular binding of calcium occured, shows that at 55°C the cellular calcium concentration represents a 50-fold increase over the medium level.

The Arrhenius plot in fig.4 indicates that the calcium uptake rate increases with temperature, in accordance with the higher demand for calcium at elevated temperatures. The temperature coefficients for the linear range 50°C to 60°C are for viability 0.51 ± 0.04 and for uptake 0.67 ± 0.07. The difference is significant at the 5% level and allows for an increase in calcium content per living cell with temperature. Note the comparably rapid increase in uptake relative to viability between 45°C and 50°C. This range corresponds to the boundary between bacterial mesophilism and thermophilism [9], and this picture could be expected if calcium transport was the master reaction controlling life in the thermophilic range. The uptake rate at 65°C appears not to be sufficient to maintain a correspondingly high growth rate, which might mean that the maximum stabilization due to calcium occurs in the range 60°C to 65°C.

Thus calcium appears to be taken up by living cells at a rate which increases with temperature. In the light of earlier results [6,7] that calcium is

needed for survival at high temperatures, we conclude that calcium is not just accumulated as a consequence of, but is indeed required for thermophilic growth of *B. stearothermophilus.*

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