Influence of Divalent Cations on the Growth and Morphology of *Bacillus stearothermophilus*

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Bacillus stearothermophilus grows within the temperature range 40–70 °C in a complex medium that contains 115 µM-Ca²⁺ and 95 µM-Mg²⁺. The addition of Ca²⁺ to a final concentration ranging from 2.5 to 10 mM stimulated growth at suboptimal and supraoptimal temperatures, extending growth above 70 °C, but had no effect on growth within the optimal temperature range. Mg²⁺ (2.5 mM) also stimulated growth although to a lesser extent. Furthermore, 10 mM-Mg²⁺ inhibited growth at temperatures higher than 65 °C. This inhibitory effect was relieved by the addition of 2.5 mM-Ca²⁺. Sr²⁺ (10 mM), which often behaves as a Ca²⁺ analogue in biological systems, strongly inhibited growth and produced gross morphological alterations in the cells. The inhibitory effect of Sr²⁺ could also be relieved by addition of Ca²⁺.

INTRODUCTION

Several mechanisms have been proposed to explain the ability of thermophilic micro-organisms to grow at temperatures at which mesophilic (prokaryotic and eukaryotic) organisms can not survive. The capacity of thermophiles to grow at higher temperatures than mesophiles is generally regarded as a consequence of their ability to synthesize cell components with increased heat stability (Friedman, 1968; Irwin *et al.*, 1973; Singleton & Amelunxen, 1973). Several investigations emphasize the importance of membrane stability in relation to heat tolerance; Brock (1967) has suggested that the molecular mechanism of thermophily is possibly more related to the function and stability of the membrane than to any special macromolecule. The capacity of thermophiles to control the physical properties of the cytoplasmic membrane by changing its lipid composition in response to temperature changes has been supported by the work of several authors (Daron, 1970; Ray *et al.*, 1971*a, b*; Weerkamp & Heinen, 1972; Hasegawa *et al.*, 1980). Ions, especially divalent cations also have a role in thermostability (Ljunger, 1970; Ståhl & Ljunger, 1976; Ståhl, 1978; Mosley *et al.*, 1976). Ljunger (1970) has proposed that Ca²⁺, accumulated inside the cell by active transport, is required for the thermostability of cell proteins. However, Mosley *et al.* (1976) suggested that divalent cations increase membrane stability by the formation of cation-bridges between the anionic groups of the acidic membrane phospholipids.

The present study reports the effect of the divalent cations Ca²⁺, Mg²⁺ and Sr²⁺ on growth and morphology of *Bacillus stearothermophilus* at different temperatures.

METHODS

Organism and culture conditions. The strain of *Bacillus stearothermophilus* used was isolated from discs impregnated with spores supplied by Mast Laboratories, UK (lot no. 8879). Stock cultures were maintained at 4 °C on tryptic soy agar (Difco) slants.

Cultures were grown in dilute L-broth containing 5.0 g D-glucose, 5.0 g tryptone (Difco), 2.5 g yeast extract (Difco), 1.25 g NaCl and 0.024 g L-cystine. HCl in 1 l water (final pH 7.0–7.2); 15 g Difco-Agar was added for solid

medium. Concentrated stock solutions of CaCl_2 , MgCl_2 and SrCl_2 (nominally 1 M) were sterilized by filtration and added to the growth medium to give the desired final cation concentrations.

Liquid cultures were started with an early stationary phase inoculum from dilute L-broth without added cations, grown within the optimal temperature range, to give an initial OD_{610} of about 0.03. Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium shaken at 130 r.p.m. in a New Brunswick (RW-650) water bath shaker; growth was measured as optical density, using a Varian Techtron spectrophotometer at 610 nm, or by counting c.f.u. on agar plates.

Analytical methods. Magnesium and calcium were determined in the control medium without added ions and in the stock solutions by atomic absorption spectrometry using a Perkin-Elmer model 305 spectrometer. Interference by phosphates was controlled by including 0.5% La^{3+} in standards and samples.

Electron microscopy. Broth cultures collected by centrifugation (6000 g for 10 min at 4 °C) were pre-fixed in 2.5% (w/v) glutaraldehyde buffered with cacodylate (0.2 M, pH 7.2) supplemented with 10 mM- CaCl_2 , for 1 h at room temperature (Silva, 1984). The cells were then fixed overnight in 1% (w/v) OsO_4 buffered with veronal/acetate (Ryter & Kellenberger, 1958) pH 6.2, supplemented with 10 mM- CaCl_2 . Post-fixation was done with 0.5% (w/v) uranyl acetate in water for 1 h. After dehydration, the material was embedded in Epon resin and ultrathin sections were obtained. The sections were contrasted with lead citrate according to Venable & Coggeshall (1965) and viewed in a Joel 100S electron microscope at 80 kV.

RESULTS

Effect of divalent cations on growth at different temperatures

The strain of *B. stearothermophilus* used in this study grew in the temperature range 40–70 °C in a complex medium (dilute L-broth) with endogenous concentrations of 0.115 mM- Ca^{2+} and 0.095 mM- Mg^{2+} (control medium).

At supraoptimal temperatures (above 65 °C), growth in control medium was characterized by a progressively longer lag period, a lower specific growth rate and a lower final culture density. This trend could be relieved by addition of Ca^{2+} (Fig. 1). The stimulatory effect of Ca^{2+} on growth was concentration dependent between 0.25 and 2.5 mM; higher concentrations of Ca^{2+} (up to 10 mM) had no additional effect on growth. The experiments performed at 70 °C also indicated that growth in the control medium was erratic, occurring in some experiments but not in others, probably reflecting slight changes in the temperature of incubation.

Addition of 2.5 mM- or 10 mM- Ca^{2+} to cultures at supraoptimal temperatures increased the growth rate and permitted significant growth at temperatures up to 72 °C (Table 1). In contrast to this effect at supraoptimal temperatures, no effect was observed between 55 and 65 °C. Ca^{2+} also stimulated the growth rate at suboptimal temperatures, but to a lesser extent than was observed at supraoptimal temperatures.

Mg^{2+} at a concentration of 2.5 mM stimulated growth at suboptimal and supraoptimal temperatures, but to a lesser extent than Ca^{2+} . A slight stimulatory effect of 2.5 mM- Mg^{2+} was observed between 68 and 71 °C, but at 72 °C this concentration of Mg^{2+} , in contrast to Ca^{2+} , had no detectable effect on growth within the experimental period. At the suboptimal temperature of 45 °C, 2.5 mM- and 10 mM- Mg^{2+} also caused a slight increase in growth rate but, as in the case of Ca^{2+} , no effect was detected between 55 and 65 °C. In contrast to 10 mM- Ca^{2+} , which stimulated growth above the optimal temperature range, 10 mM- Mg^{2+} had a profound inhibitory effect at temperatures above 65 °C (Table 1). The inhibition of growth induced by 10 mM- Mg^{2+} was reversed when 2.5 mM- or 10 mM- Ca^{2+} was added to the culture, resulting in a similar growth rate to the 2.5 mM- or 10 mM- Ca^{2+} control cultures (Fig. 2).

The addition of low concentrations of Sr^{2+} (0.25 and 0.5 mM) had no effect on growth, while 2.5 mM- and 10 mM- Sr^{2+} had a generalized inhibitory effect on growth at temperatures above 60 °C. The inhibition of growth induced by Sr^{2+} was reflected in a decrease both in the growth rate (Table 1) and in the final cell density. Often, the cultures with 2.5 mM- Sr^{2+} had a high initial growth rate followed by a lower rate as growth proceeded. The inhibitory effect of 10 mM- Sr^{2+} was more profound and at low temperatures (i.e. 60 °C) was characterized by a fairly high initial growth rate followed by an abrupt cessation of growth and a decrease in the number of c.f.u. (Fig. 3a). At higher temperatures, growth in medium with 10 mM- Sr^{2+} was initially noticeable, but ceased thereafter (Fig. 3b). The inhibitory effect of 10 mM- Sr^{2+} on growth could be relieved, as for Mg^{2+} -inhibited cultures, by the addition of 2.5 mM- Ca^{2+} for at least 2 h after inoculation of the cells.

Fig. 1

Fig. 2

Fig. 1. Effect of Ca^{2+} addition on the growth of *B. stearrowtherophilus* at 70 °C in control medium (○) and media containing Ca^{2+} at a final concentration of 0.25 mM (■), 1.0 mM (□), 2.5 mM (●), 5 mM (△) and 10 mM (▲). The results shown are typical of those obtained in three separate experiments.

Fig. 2. Effect of Ca^{2+} addition to cultures of *B. stearrowtherophilus* inhibited by 10 mM- Mg^{2+} at 71 °C. Growth was measured by determining the number of c.f.u. in media containing 2.5 mM- Ca^{2+} (△), 10 mM- Mg^{2+} (■), and 10 mM- Mg^{2+} to which 2.5 mM- Ca^{2+} was added (arrow) 1 h after inoculation (●). The results shown are typical of those obtained in two separate experiments.

Table 1. Specific growth rates (h^{-1}) of *B. stearrowtherophilus* in dilute L-broth and in the same medium supplemented with Ca^{2+} , Mg^{2+} or Sr^{2+}

Growth temp. (°C)	Specific growth rate* (μ) in dilute L-broth with:			
	No added cations	10 mM- Ca^{2+}	10 mM- Mg^{2+}	10 mM- Sr^{2+}
45	0.15 $\pm$ 0.07 (3)	0.58 (1)	0.59 (1)	ND
50	1.24 $\pm$ 0.02 (2)	1.47 $\pm$ 0.10 (2)	1.35 $\pm$ 0.07 (2)	1.46 (1)
55	2.32 $\pm$ 0.02 (2)	2.44 $\pm$ 0.06 (2)	2.35 $\pm$ 0.11 (2)	2.22 (1)
60	2.92 $\pm$ 0.22 (7)	3.04 $\pm$ 0.28 (2)	3.15 $\pm$ 0.06 (2)	2.25 $\pm$ 0.13 (4)
65	2.91 $\pm$ 0.20 (5)	3.05 $\pm$ 0.05 (3)	2.87 $\pm$ 0.06 (3)	1.79 $\pm$ 0.00 (2)†
68	2.58 $\pm$ 0.21 (5)	2.70 $\pm$ 0.33 (2)	1.77 $\pm$ 0.18 (3)	1.86 $\pm$ 0.55 (5)†
70	0.0 (3)‡		0.0 (3)‡	0.0 (3)‡
	1.04 $\pm$ 0.48 (10)	2.10 $\pm$ 0.17 (8)	0.58 $\pm$ 0.13 (2)	0.26 (1)†
71	0.0 (17)‡			
	0.23 $\pm$ 0.04 (3)	1.49 $\pm$ 0.13 (7)	0.0 (12)	0.0 (3)
72	0.0 (3)	0.37 $\pm$ 0.41 (3)	0.0 (1)	ND
73	0.0 (1)	ND	ND	ND

ND, Not determined.

* Mean $\pm$ standard deviation, with the number of experiments in parentheses.

† Maximal bacterial concentrations in these conditions were very low in spite of the relatively high specific growth rates.

‡ Growth at these temperatures was erratic, occurring in some experiments but not in others.

Fig. 3. Effect of Sr^{2+} on the growth of *B. stearothermophilus*. (a) Growth (determined as c.f.u.) of cultures grown at 60 °C in media containing 2.5 mM- Ca^{2+} (●), 2.5 mM- Sr^{2+} (▲) and 10 mM- Sr^{2+} (△). (b) Reversal of the inhibition by Sr^{2+} was determined by adding 2.5 mM- Ca^{2+} (arrow) to a culture grown in medium containing 10 mM- Sr^{2+} at 68 °C (▲). Growth was also monitored in control medium (○), and media containing 10 mM- Ca^{2+} (●), 10 mM- Mg^{2+} (■) and 10 mM- Sr^{2+} (△). The results shown are typical of those obtained in two separate experiments.

Formation of abnormal cells in Sr^{2+} -containing medium

Phase-contrast and electron microscopy revealed morphological alterations in cells of *B. stearothermophilus* grown in media containing 2.5 mM- and 10 mM- Sr^{2+} . Bacteria were stained with crystal violet and measurements of 50 representative cells were made using a stage micrometer. Early stationary phase cells at 60 °C derived from medium containing 2.5 mM- Ca^{2+} were $2.24 \pm 0.95 \mu\text{m}$ long and $0.48 \pm 0.05 \mu\text{m}$ wide. The addition of 2.5 mM- Sr^{2+} instead of Ca^{2+} resulted in the formation of slightly abnormal cells in which the major difference detected by phase-contrast microscopy was an increase in the cell width to $1.02 \pm 0.13 \mu\text{m}$. Much larger cells, $6.05 \pm 1.92 \mu\text{m}$ long and $1.61 \pm 0.45 \mu\text{m}$ wide, were observed in cultures containing 10 mM- Sr^{2+} . These cells also displayed aberrant shapes, contrasting with the regular rod-shaped cells grown in control medium or media containing added Ca^{2+} or Mg^{2+} . Sr^{2+} -inhibited cultures also contained cells undergoing lysis and abundant cellular debris. Formation of abnormal cells in cation-inhibited cultures was induced only by Sr^{2+} : growth inhibition by 10 mM- Mg^{2+} did not induce abnormal morphologies. Electron microscopy of the Sr^{2+} -inhibited cells showed incomplete septa and enlargement of the cell dimensions, but the cell wall retained the thickness characteristic of cells grown in control medium or media containing added Ca^{2+} (Fig. 4).

DISCUSSION

Earlier investigations have shown that divalent cations are required for thermophilic growth (Ljunger, 1970; Ståhl & Ljunger, 1976; Ståhl, 1978). The growth dependence of the mesophile *Escherichia coli* on divalent cations has also been reported (Arancia *et al.*, 1980): when divalent cations were limiting, the lag periods and doubling times were longer and the final bacterial concentrations were lower than in medium supplemented with 1 mM- Mg^{2+} ; a sharp fall in viable

Fig. 4. Electron micrographs of *B. stearrowthermophilus* from early stationary phase cultures at 65 °C: (a) in medium containing 2.5 mM- Ca^{2+} , (b) in medium containing 2.5 mM- Sr^{2+} and (c, d) in medium containing 10 mM- Sr^{2+} . Bars, 1 μm .

counts was also observed at the end of the exponential growth phase. Our results with *B. stearrowthermophilus* are essentially consistent with these earlier observations. Thus, Ca^{2+} improved growth at supraoptimal temperatures, inducing higher specific growth rates, higher bacterial yields and shorter lag phases. Furthermore, Ca^{2+} extended the range of the maximal temperature for growth.

The effect of Mg^{2+} on the growth of *B. stearrowthermophilus* differed significantly from that of Ca^{2+} in that 10 mM- Mg^{2+} , in contrast to 2.5 mM- Mg^{2+} (which was stimulatory), inhibited growth

at supraoptimal temperatures. This effect was never observed with the same concentration of Ca^{2+} . An effect of Mg^{2+} concentration on the growth of different mesophilic bacteria in complex media was observed by Webb (1966): at low concentrations, Mg^{2+} stimulated growth, but above a critical concentration, growth was progressively inhibited. Our studies with *B. stearothermophilus* additionally showed that the inhibitory effect of Mg^{2+} could be reversed by addition of Ca^{2+} . Presumably Ca^{2+} displaces Mg^{2+} at the divalent cation interaction sites and thus relieves the inhibitory effect.

Sr^{2+} is commonly believed to behave in certain biological systems as a Ca^{2+} analogue; thus, calcium pumps do not distinguish between Ca^{2+} and Sr^{2+} (Olson & Cazort, 1969; Mermier & Hasselbach, 1976; Guimarães-Motta *et al.*, 1984). However, in our studies Sr^{2+} and Ca^{2+} had readily distinguishable effects on the growth of *B. stearothermophilus*. Sr^{2+} , in contrast to Ca^{2+} , strongly inhibited growth and induced dramatic changes in cell morphology including uncontrolled increase in cell size, leading eventually to cell lysis. As with Mg^{2+} , inhibition of growth by Sr^{2+} was relieved by addition of Ca^{2+} , suggesting competition of Sr^{2+} and Ca^{2+} for the same interaction sites, presumably located at external, readily accessible structures such as the membrane.

Morphological alterations like those we observed in *B. stearothermophilus* in the presence of $>2.5 \text{ mM-Sr}^{2+}$ have been described in *E. coli* and *Bacillus subtilis* mutants (Long *et al.*, 1978; Rogers & Thurman, 1978) and in *Bacillus caldolyticus* (Aart *et al.*, 1981). These alterations were believed to reflect various environmental stresses such as changes in oxygen, nitrogen or glucose concentrations, pH or temperature alterations and the presence of drugs (6-amidinopenicillanic acid) or certain chemicals, such as nitrate and halides (Long *et al.*, 1978; Rogers & Thurman, 1978). *E. coli* grown in a medium deficient in divalent cations showed structural modifications in the cytoplasmic membrane following a presumed lipid phase transition, not observed in bacteria grown in control medium containing 1 mM-Mg^{2+} (Arancia *et al.*, 1980). The structural integrity of protoplast membranes of *B. stearothermophilus* was also shown to be maintained by divalent cations (Wisdom & Welker, 1973). Studies are now in progress with liposomes and protoplasts of *B. stearothermophilus* to ascertain the effect of Ca^{2+} , Mg^{2+} and Sr^{2+} on the biophysical properties of their membranes.

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