JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 150-155

Vol. 105, No. 1
Printed in U.S.A.

Evidence for a Magnesium Pump in *Bacillus cereus* T

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Received for publication 29 September 1970

Unlike *Escherichia coli*, *Bacillus cereus* T appears to accumulate Mg^{2+} in its cell sap against a concentration gradient. Over a range of Mg^{2+} in the growth medium from 5×10^{-5} to 1.35×10^{-2} M, the concentration of Mg^{2+} in the cell sap of *B. cereus* T was maintained at about 6×10^{-3} M, and ribosome-bound Mg^{2+} and spermidine, as well as the spermidine concentration in the cell sap, appear to be unaffected by the concentration of Mg^{2+} in the growth medium. Inhibition of growth of *E. coli* by streptomycin is progressively reversed by increasing the concentration of Mg^{2+} in the growth medium above 5 mM. The finding that similar increases of Mg^{2+} in the growth medium did not reverse the inhibition of *B. cereus* T is also consistent with the conclusion that *B. cereus* T, unlike *E. coli*, accumulates Mg^{2+} to a constant concentration in its cell sap.

We previously reported that *Escherichia coli* ML35 (11) and other gram-negative organisms (16) contain the same concentration of Mg^{2+} in their cell sap as is present in the growth medium over a range of Mg^{2+} in the latter from 4×10^{-6} to 10^{-2} M. The technique used was based on the separation of cells from the aqueous medium by centrifugation through a layer of silicones more dense than the aqueous medium but less dense than the bacteria (10). The recovered cellular pellets were then diluted with an equal volume of water and disrupted by passage through a French pressure cell. Since the concentration of Mg^{2+} in the recovered high-speed supernatant fractions was found to be the same as in the growth medium over a range of 4×10^{-6} to 10^{-2} M, we concluded that these gram-negative cells do not accumulate Mg^{2+} in their cell sap against a concentration gradient. The concentration of ribosome-bound Mg^{2+} under these conditions varied directly, but not linearly, with Mg^{2+} concentration in the cell sap, whereas ribosome-bound spermidine varied inversely with ribosome-bound Mg^{2+} (11).

Bacilli appear to differ from gram-negative rods in their requirement for Mg^{2+} and in their content of polyamines. The lower limit of Mg^{2+} concentration required for growth of bacilli is appreciably higher than for gram-negative rods (20), and putrescine, which is more abundant in *E. coli* than is spermidine, is lacking or very low in bacilli (9). Since there appeared to be differences in requirement for Mg^{2+} and in content of polyamines between gram-negative rods and bacilli, we examined the effect of concentration of Mg^{2+} in the growth medium on growth of *Bacillus cereus* T, on the Mg^{2+} content of its cell sap, and on ribosome-bound Mg^{2+} and polyamines. The following data indicate that, unlike *E. coli*, *B. cereus* T has an active transport system for Mg^{2+}.

MATERIALS AND METHODS

Preparation of spore suspension. *B. cereus* T was obtained from A. Keynan. Spores, germinating spores, vegetative cells, and sporulating cells differ from one another in physiology, morphology, and cellular content (6, 7). Since comparisons were to be made with exponentially growing gram-negative cells, it was considered important to obtain vegetative cells of *B. cereus* T free of other growth stages. All studies were therefore performed with a uniform outgrowth of spores prepared by growth of *B. cereus* T at room temperature in well-aerated G medium (19). After aseptic collection by centrifugation, the pellets were washed three times with sterile distilled water, suspended in one-tenth the original volume, activated by heating at 70 C for 15 min (4, 13), and then stored at −20 C. Under these conditions there is no reversal of heat activation (12). The viable spore titer was $1.5 \pm 0.5 \times 10^8$ per ml.

Growth and collection of cells. The growth medium was that described by Aronson and Wermus (1) with the following modifications. The K_2HPO_4 and citrate concentrations were reduced to one-half to decrease the density of the medium below that of the silicone mixture. Casamino Acids (0.2%, Difco) replaced the amino acids added to improve growth, and $MgSO_4 \cdot 7H_2O$ was replaced by Na_2SO_4 and $MgCl_2 \cdot 6H_2O$ to facilitate variation of Mg^{2+} concentration. Glucose and Casamino

Acids were sterilized by filtration through membrane filters (Millipore Corp., Bedford, Mass.) and were added aseptically to the autoclaved medium after cooling.

Culture flasks containing 1.5 liters of medium were inoculated with 7×10^2 to 7×10^3 spores/liter and were rotated at 30 C in a gyratory water bath (New Brunswick Scientific Co., New Brunswick, N.J.). Growth was measured in nephelos units in a Coleman model 7 Nephelometer. At 400 nephelos units, the culture contained 0.246 mg of dry weight, 0.109 mg of protein, and 0.054 mg of ribonucleic acid (RNA) per ml. Cultures were grown to middle log phase (310 to 440 nephelos units). Microscopic examination (phase contrast) served as a check for presence of other than fully grown vegetative cells. Cultures were rapidly chilled to 4 C in a -20 C ethylene glycol bath to arrest growth.

Cells were harvested in a continuous-flow apparatus at 12,000 $\times$ *g* in a Sorvall RC2-B refrigerated centrifuge. A 15-ml amount of an ice-cold mixture of silicone fluids was placed in cellulose nitrate tubes (1 by 3 inches) and carefully covered with a protective collar of 15 ml of ice-cold distilled water to prevent flushing out of silicones at the onset of centrifugation. The cells were collected in these tubes by continuous-flow centrifugation through the mixture of silicone fluids designed to be more dense than the growth medium, but less dense than the bacteria (10). The silicone procedure was used to obviate an aqueous wash of the cells which resulted in considerable loss of intracellular Mg^{2+}. Pellets were drained briefly, pooled, and weighed. Examination of the pellet by phase-contrast microscopy revealed that, as with *E. coli* (10), some entrained medium accompanied the cells through the silicone layer.

Estimation of entrained medium. A direct method for determination of the fractional distribution of extra- and intracellular water in a cellular pellet can be obtained by a modification of the procedure used by Harris and vanDam (8), in which the total water in mitochondria is measured as that which is exchangeable with tritiated water and the extracellular water is measured as that which will dilute a solution of molecules which does not penetrate the cellular membrane.

Pellets of *B. cereus* T were collected in triplicate by centrifugation through a layer of silicones under the same conditions as described earlier. Each sample contained the cells from about 80 ml of growth medium. To each pellet was added 80 μliters of a solution containing 0.16 μCi of tritiated water and 0.013 μCi of dextran-carboxyl-^{14}C (molecular weight 15,000–17,000). After thorough mixing, each suspension was centrifuged in a 1-ml conical tube for 30 min at 12,800 $\times$ *g*. Three to four 10-μliter samples of the supernatant fluid were added to 10 ml of Bray's solution, and radioactivity was measured in a model 3375 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). The total water space in the pellet was calculated from the decrease in specific activity per unit volume of ^{3}H, and the extracellular water space was calculated from the decrease in specific activity per unit volume of ^{14}C-dextran solution. The difference between the two calculated volumes was taken as measurement of the intracellular water space (cell sap). The fractional amount of extracellular water was found, by this method, to be 0.481 $\pm$ a mean deviation of 0.013.

Cell disruption and fractionation. An equal volume of water was added to the pooled pellets, and the cells were disrupted by being passed twice through a prechilled French pressure cell (American Instrument Co., Silver Spring, Md.) at 10,000 to 15,000 psi. A 1-ml amount of the disrupted cell suspension was then incubated with deoxyribonuclease (0.02 ml of 300 μg/ml in 0.1 M acetate buffer, *p*H 5.1) for 5 min at room temperature and then centrifuged at 20,000 $\times$ *g* for 20 min. The supernatant fluid was further clarified by centrifugation at 30,000 $\times$ *g* for 30 min. This latter supernatant fluid (S-30 fraction) was then centrifuged for 2 hr in a Spinco model L ultracentrifuge at 100,000 $\times$ *g*. The supernatant fluid from centrifugation at 100,000 $\times$ *g* consisted of a clear upper layer and a cloudy lower layer, and was further centrifuged at 100,000 $\times$ *g* for 4 hr without brake to better separate the two layers. The upper clear layer is designated as the S-100 fraction. Another small ribosomal pellet was obtained after this last centrifugation but was not combined with the original ribosomal pellet.

The lower cloudy layer of the high-speed supernatant fluid was partially analyzed. It contained protein (4 mg/ml), RNA (0.25 mg/ml), and lipids. β-Hydroxybutyrate was present in only trace amounts, if at all. No glycogen was found by using an anthrone reagent assay (18).

An apparently similar, although smaller, layer in *E. coli* cells has been shown to contain ribosomes, RNA, protein, and phospholipids and has been suggested to consist of ribosomes attached to fragments of cellular membrane (11).

Magnesium ion assay. Mg^{2+} assays were performed by the Schachter method (17) on 0.1 ml of a 1:10 dilution of the S-100 fractions and 0.05 ml of a 1:10 dilution of suspended ribosomes. Clark and Hou (3) noted that phosphate ions in concentrations above 5 meq/liter interfere in this method. The above dilution of the S-100 fractions obviated removal of phosphates from undiluted samples which required passage through a Dowex-1-Cl^- exchange resin. Recovery experiments showed that the dilution used also effectively eliminated interference by Ca^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+} (3).

Polyamine and RNA analyses. Polyamines were solubilized in ice-cold trichloroacetic acid and determined as described previously (11). RNA was determined in the cold, acid-precipitated fraction by the orcinol method (15).

RESULTS

Growth curves as a function of Mg^{2+} concentration. Samples (50 ml) of medium at various concentrations of Mg^{2+} in 300-ml side-arm flasks were inoculated with about 8×10^6 spores per flask. The growth curves obtained are shown in Fig. 1. As Mg^{2+} concentration decreased below 0.05 mM, the lag time before onset of growth increased markedly. Total growth also decreased as the concentration of Mg^{2+} decreased, and some

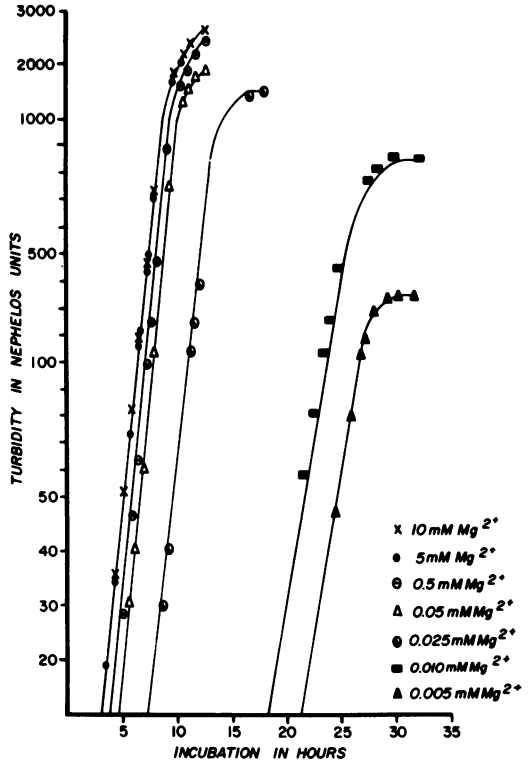

FIG. 1. *Effect of concentration of Mg^{2+} on outgrowth of spores of Bacillus cereus T. Growth was measured as increase in turbidity at 30 C. Turbidity was measured in nephelos units with a Coleman model 7 Nephelometer. The concentrations of Mg^{2+} given are those at the time of inoculation. A suspension of B. cereus T with a reading of 400 nephelos units contains (per ml): 0.246 mg of dry weight, 0.109 mg of protein, and 0.054 mg of RNA.*

decrease in growth rate was observed below 0.025 mM Mg^{2+}.

Magnesium ion concentration in the cell sap. Mg^{2+} concentration in the S-100 fractions of cells grown at various concentrations of Mg^{2+} in the growth medium were assayed as described in Materials and Methods. As indicated previously, the S-100 fractions consist of entrained medium, cell sap, and the water added to enable the collected cells to be disrupted. Table 1 lists the values for Mg^{2+} in the uncorrected S-100 fractions, after correction for added water and after further correction for Mg^{2+} in entrained medium. The corrections consist of (i) multiplying the concentrations of Mg^{2+} in the S-100 fractions by 2.25 to correct for water added to the pellet, and (ii) then subtracting the Mg^{2+} in the entrained medium, which was found to comprise 48% of the total water in the pellet collected under silicones by centrifugation.

TABLE 1. *Concentration of Mg^{2+} in S-100 fractions of B. cereus T*

Mg^{2+} in growth medium (mM)	mM Mg^{2+} in S-100 Fractions		
	Uncorrected	Corrected for added water[a]	Corrected for entrained medium[b]
13.5	4.83 ± .50	10.88	8.46
8.9	2.98 ± .15	6.70	4.67
7.0	2.61 ± .15	5.86	6.46
5.0	2.74 ± .06	6.16	7.23
0.5	1.34 ± .41	3.02	5.35
0.05	1.18 ± .33	2.66	5.07

[a] These values are referred to in the text as those of the partially corrected S-100 fractions. The uncorrected values were corrected for water added to the cellular pellets, to enable disruption in a French pressure cell, by multiplying by 2.25, since 1.0 ml of water was added per g (wet weight) of pellet which contained 80% water.

[b] Since the fractional amount of entrained medium in a pellet was 0.48, the concentration of Mg^{2+} in the cell sap was determined from the following relationship: concentration in the partially corrected S-100 fraction = 0.48 (concentration in the growth medium) + 0.52 (concentration in the cell sap).

Since the variation in Mg^{2+} content of the six cell saps appears to represent experimental error, the concentration of Mg^{2+} in the cell sap of *B. cereus* T is taken to be 6.21 mM ± a mean deviation of 1.18 over the range of 0.05 to 13.5 mM Mg^{2+} in the growth medium. This finding indicates that Mg^{2+} is accumulated against a concentration gradient by *B. cereus* T, and that these cells can maintain a lower internal concentration when the extracellular concentration exceeds that in the cell sap.

The validity of the correction for entrained medium is attested to by the finding that only in the range of 5 to 7 mM Mg^{2+} in the growth medium does the concentration of Mg^{2+} in the partially corrected S-100 fractions approximate the concentration in the growth medium. Since each partially corrected S-100 fraction is the sum of cell sap containing a constant concentration of Mg^{2+} and medium entrained during collection of the cells, the concentration of Mg^{2+} in the partially corrected S-100 fraction can equal the concentration in the growth medium only when the latter equals the concentration in the cell sap.

In *E. coli* (11), the concentration of Mg^{2+} in the partially corrected S-100 fractions was the same as that in the growth medium at all concentrations from 4×10^{-6} to 10^{-2} M in the medium. It therefore appears that *B. cereus* T, unlike *E. coli*, accumulates Mg^{2+} in its cell sap against a concentration gradient, and that *E. coli*, unlike *B. cereus* T, is freely permeable to Mg^{2+}.

Since the concentration of Mg^{2+} in the cell sap

of *B. cereus* T appears to be constant at about 6 mM, whereas the concentration in the cell sap of *E. coli* appears to vary with that of the growth medium, it seemed reasonable to expect concomitant differences in ribosome-bound Mg^{2+} and ribosome-bound spermidine in the two organisms. In *E. coli*, where the Mg^{2+} concentration in the cell sap varied widely (11), the amount of ribosome-bound Mg^{2+} was proportional to the concentration of Mg^{2+} in the growth medium, and the amount of ribosome-bound spermidine was inversely proportional to the amount of ribosome-bound Mg^{2+}. One would therefore expect that, if the Mg^{2+} concentration in the cell sap of *B. cereus* T is constant at all extracellular concentrations, the amount of ribosome-bound Mg^{2+} and spermidine should be unaffected by varying concentrations of Mg^{2+} in the growth medium. Table 2 shows that ribosome-bound Mg^{2+} and ribosome-bound spermidine in *B. cereus* T are both unaffected by the concentration of Mg^{2+} in the growth medium. The amount of spermidine in the cell sap of *B. cereus* T, which is considerably higher in this organism than in *E. coli*, is also unaffected by the concentration of Mg^{2+} in the medium (Table 3).

Effect of Mg^{2+} on inhibition of growth by streptomycin. It has been shown that the inhibiting action of streptomycin in gram-negative organisms can be progressively reversed by increasing the concentration of Mg^{2+} in the growth medium (2, 5, 14). If *E. coli* is freely permeable to Mg^{2+}, and if entry of Mg^{2+} into *B. cereus* T is mediated by an active transport process, one would expect that streptomycin inhibition of *E. coli* would be reversed by increasing external concentration of Mg^{2+}, whereas streptomycin inhibition of *B. cereus* T would be unaffected.

To test this hypothesis, *E. coli* ML35 and *B. cereus* T were both grown in medium G at 5 mM Mg^{2+}. Under these conditions, it was found that 40 μg of streptomycin/ml would inhibit growth of either cell type substantially but not completely. When the Mg^{2+} content of the growth medium was increased to 20 mM, the streptomycin inhibition of *E. coli* ML35 was almost completely reversed, whereas the inhibition of *B. cereus* T was unaffected (Fig. 2, 3).

TABLE 2. *Ribosome-bound Mg^{2+} and spermidine in B. cereus T*

Mg^{2+} in growth medium (mM)	Ribosome-bound Mg^{2+}/ ribosomal RNA-ribose (μmole/μmole)	Ribosome-bound spermidine/ ribosomal RNA-ribose (μmole/μmole)
13.5	0.168 ± 0.001	
7.0	0.150 ± 0.001	
5.0	0.126 ± 0.003	0.0412 ± 0.0015
0.5	0.124 ± 0.004	0.0384 ± 0.0055
0.05	0.095 ± 0.008	0.0547 ± 0.0250

TABLE 3. *Concentration of spermidine in S-100 fractions of B cereus T*

Mg^{2+} in growth medium (mM)	Spermidine in S-100 fractions	
	Partially corrected[a] (mM)	Fully corrected[b] (mM)
5.00	0.571 ± .098	1.10
0.50	0.470 ± .203	0.90
0.05	0.876 ± .309	1.68

[a] Corrected for dilution with water added to the cellular pellet to enable disruption of cells in a French pressure cell (*see* Table 1)

[b] Further corrected for medium entrained in the cellular pellet during centrifugation. Since there was no spermidine in the entrained medium, the correction for dilution with entrained medium is obtained by dividing the partially corrected concentrations by 0.52, the fractional proportion of the cell sap in the total water of the cellular pellet.

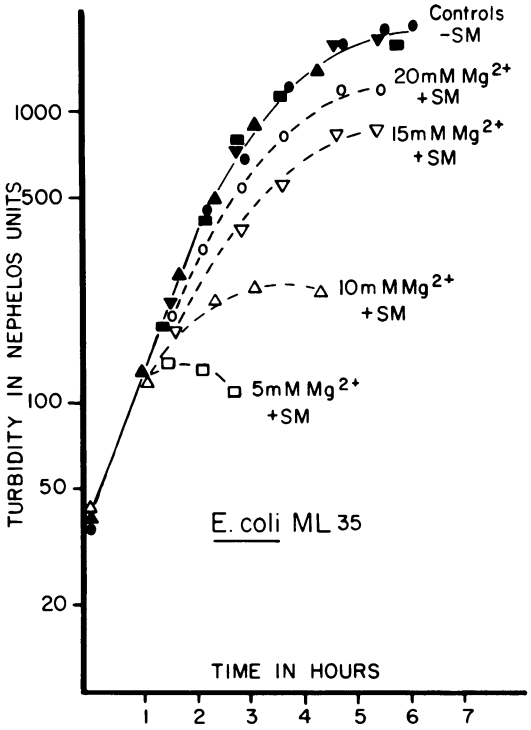

FIG. 2. *Effect of concentration of Mg^{2+} on inhibition of growth of Escherichia coli ML35 by streptomycin. Streptomycin (SM) at 40 μg/ml was added to cells growing exponentially at about 5 × 10^7/ml in the same minimal medium used for vegetative growth of B. cereus T. Growth was measured in a Coleman model 7 Nephelometer. See Fig. 1 for definition of nephelos units.*

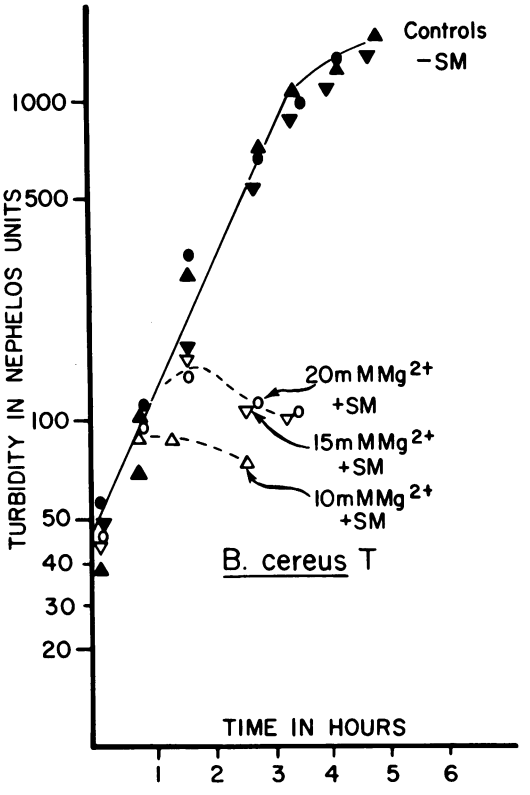

FIG. 3. *Effect of concentration of Mg^{2+} on inhibition of growth of B. cereus T by streptomycin. Conditions were the same as for the experiment described in Fig. 2. Streptomycin (SM) at 40 μg/ml was added when the vegetative growth reached a turbidity of 40 nephelos units.*

DISCUSSION

By using the same procedures as with *E. coli*, i.e., washing the cells by centrifugation through silicones and determining the concentration of Mg^{2+} in the cell sap after disruption of the cells by passage through a French pressure cell, we found that *B. cereus* T, unlike *E. coli*, maintains Mg^{2+} in its cell sap at about 6 mM against a concentration gradient.

That the two cell types differ in this respect is further documented by the ancillary findings that, in *B. cereus* T, ribosome-bound Mg^{2+} and spermidine and intracellular concentration of spermidine are not affected by external concentration of Mg^{2+}. In *E. coli* ML35, ribosome-bound Mg^{2+} varies directly with extracellular (and intracellular) Mg^{2+}, whereas ribosome-bound spermidine varies inversely with ribosome-bound Mg^{2+} (11).

Our finding that an increase in external concentration of Mg^{2+} reverses streptomycin inhibition of *E. coli* but not of *B. cereus* T is also consistent with the view that *E. coli* is freely permeable to Mg^{2+}, whereas Mg^{2+} enters *B. cereus* T by means of an active transport process.

The finding that *B. cereus* T maintains Mg^{2+} at a concentration of approximately 6 mM in its cell sap, whereas *E. coli* appears to be able to grow optimally at concentrations of Mg^{2+} below 10^{-2} mM in its cell sap (12), suggests that these two cell types differ markedly in their requirement of Mg^{2+} and of polyamines for protein synthesis.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-07737 from the National Institute of Allergy and Infectious Diseases.

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