

# Potassium Content During Growth and Sporulation in *Bacillus subtilis*

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Vegetative and sporulating cells of *Bacillus subtilis* retain a higher level of internal potassium than do nonsporulating stationary-phase cells. The addition of manganese to nonsporulating stationary-phase cells, at concentrations required for sporulation, rapidly stimulates uptake and net accumulation of potassium and induces sporulation.

Bacteria such as *Escherichia coli* and *Bacillus subtilis* accumulate potassium ions against a concentration gradient during growth. The high intracellular potassium content fulfills the potassium requirement for macromolecular synthesis during growth (5, 6, 12), germination (4), and lytic infection by bacteriophage (2, 13). Cells of *E. coli* from cultures in stationary phase have a lower capacity for accumulating potassium than cells from cultures in exponential phase, and, when the cells are maintained in stationary phase, the intracellular concentration gradually drops to the extracellular concentration (8).

In the proper nutrient environment and in the presence of manganese, *B. subtilis* can undergo sporulation after it enters the stationary phase of growth. The results reported here show that sporulating cells have a potassium content similar to that of exponentially growing cells and that the addition of manganese to nonsporulating stationary-phase cells causes a rapid stimulation of potassium accumulation.

## MATERIALS AND METHODS

**Bacteria and media.** *B. subtilis* strain W23 was obtained from S. Bose, stored on nutrient broth slants at 4 C, and transferred at monthly intervals. Tryptone broth containing 8 g of tryptone (Difco) and 5 g of NaCl per liter was used for growth of vegetative and sporulating cells. The measured potassium content of tryptone broth is 0.7 mM. For sporulation, tryptone broth was supplemented with 10  $\mu$ M MnCl<sub>2</sub>. The measured magnesium, calcium, and manganese concentrations of the unsupplemented broth are 0.08 mM, 0.1 mM, and less than 0.1  $\mu$ M, respectively.

**Potassium 42 measurements.** Potassium 42 ac-

cumulation was measured as described by Silver, Levine, and Spielman (9) and Silver and Wendt (10), with slight modifications as noted in the figure legends. High-specific-activity <sup>42</sup>K (2 to 5 Ci/g) was obtained from the Cambridge Nuclear Corp., Cambridge, Mass., and from International Chemical and Nuclear, Irvine, Calif., and was measured in a Nuclear-Chicago Corp. low-background gas-flow counter.

**Protein synthesis.** Incorporation of <sup>14</sup>C-leucine into protein was determined by standard trichloroacetic acid precipitation followed by membrane filtration. <sup>14</sup>C-leucine was obtained from New England Nuclear Corp., Boston, Mass., and was added to cultures at 0.4  $\mu$ Ci/ml. Periodically, 0.2-ml samples were removed and added to 2 ml of iced, 5% trichloroacetic acid. After 3 hr on ice, the trichloroacetic acid precipitates were filtered (HA filters, Millipore Filter Corp., Bedford, Mass.) and washed with 15 ml of cold, 1% trichloroacetic acid. The dried filters were counted on planchets.

**Flame photometry.** To determine net cellular potassium, *B. subtilis* cells were harvested by centrifugation, washed once with water, and re-centrifuged. The pellets were dried overnight in an oven at 100 C. The pellets were acid hydrolyzed over a Bunsen burner with 1 ml of a 2:1 mixture of concentrated HNO<sub>3</sub> and concentrated H<sub>2</sub>SO<sub>4</sub> until the solutions cleared. After dilution into a LiCl standard solution, potassium content was measured in a model 143 flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass.).

**Growth and sporulation conditions.** Bacterial cultures were started with 50- or 100-fold dilutions of overnight cultures in unsupplemented tryptone broth and shaken in a 37 C room. The cultures usually occupied 10% or less of the flask volume. Neither stationary-phase nor sporulating *B. subtilis* W23 shows obvious cell lysis (decrease in optical density) during the first 18 hr of incubation (as occurs with other strains of *B. subtilis* under these conditions). Sporulation is asynchronous, however, and not as complete as with other strains and conditions. In the manganese-supplemented broth, mi-

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microscopically visible spores appear in 10 to 20% of the cells at about 12 hr;  $^{45}\text{Ca}$  accumulation is measurable at 9 hr (3); by 24 hr of incubation about  $2.5 \times 10^8$  heat resistant spores can be recovered per milliliter.

**Chemicals.** Chloramphenicol was a gift from Parke, Davis & Co., Detroit, Mich. Reagent grade, inorganic salts were used throughout.

## RESULTS

**Potassium content during growth and sporulation.** When *B. subtilis* W23 is grown in tryptone broth supplemented with  $10 \mu\text{M}$   $\text{Mn}^{2+}$ ,  $1 \text{ mM}$   $\text{Ca}^{2+}$ , and  $1 \text{ mM}$   $\text{Mg}^{2+}$ , the bacteria sporulate normally and yield about  $2.5 \times 10^8$  spores/ml after 24 hr of incubation. If the tryptone broth is not supplemented with these three cations, spore formation is decreased by a factor of  $10^3$ . Stationary-phase cells, from a 16-hr overnight culture in unsupplemented tryptone broth, also have a decreased potassium content (less than 0.5% of the dry weight relative to the usual 2.5 to 3% in exponential phase cells). When diluted back into fresh, unsupplemented broth or into broth supplemented with manganese, magnesium, and calcium, the cells show a net buildup of intracellular potassium during the first 3 hr of growth (exponential phase) (Fig. 1A). After the onset of stationary phase (4 to 5 hr), however, the cells cultured in unsupplemented broth begin to lose potassium (in the absence of cell lysis), whereas the cells cultured in supplemented broth retain their high potassium level throughout sporulation.

Figure 1B demonstrates that retention of high potassium levels at the end of growth depends entirely upon the added manganese. With all possible combinations of the three divalent cations, the cells maintain their high potassium levels only if manganese is present. Note that the data in Fig. 1B are expressed as potassium content per ml of cells rather than per mg of cellular dry weight as in Fig. 1A. The cell mass continues to increase between 3 and 6 hr of incubation, whereas the potassium content per unit of mass shows little change (cf. Fig. 1A and 1B).

Figure 2 shows that the addition of  $10 \mu\text{M}$   $\text{Mn}^{2+}$  to either growing or stationary-phase cells in unsupplemented broth results in the maintenance or re-establishment of the high intracellular potassium content. After the addition of manganese, net potassium content always approaches that of the cells grown in manganese throughout. The lower cellular potassium after 3 hr of growth in the culture with manganese throughout does not result from a

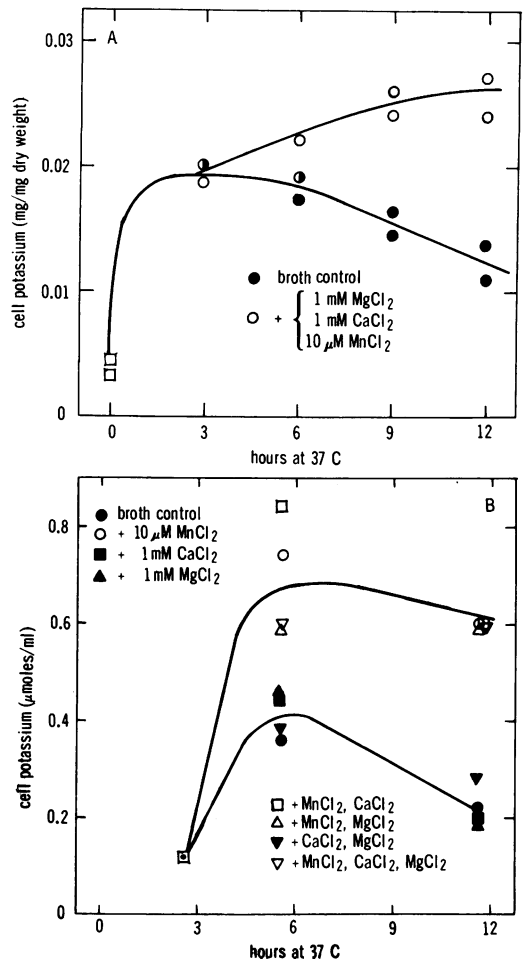


FIG. 1. Potassium content of sporulating and stationary phase *Bacillus subtilis*. (A) A 100-fold dilution from a 16-hr culture in tryptone broth was used to start cultures in broth or broth supplemented with  $10 \mu\text{M}$   $\text{MnCl}_2$ ,  $1 \text{ mM}$   $\text{MgCl}_2$ , and  $1 \text{ mM}$   $\text{CaCl}_2$ . Every 3 hr, duplicate 300- to 400-ml samples were centrifuged and used for dry weight and potassium (flame emission photometry) measurements. The initial points ( $\square$ ) at time zero are from the overnight culture used for the inoculum. (B) Culture conditions as in A, but the broth was supplemented with  $\text{MnCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{CaCl}_2$ , either singly, in pairs, or with all three salts. Cell potassium (per ml of culture) was determined by centrifuging 20 (at 3 hr) or 10 ml (all other times) of each culture and measuring by flame photometry the potassium content of the acid-extracted pellets. The symbol at 3 hr ( $\square$ ) represents the mean value from all eight samples (with a standard error of less than 10%).

lower potassium content but, rather, reflects the lower cell mass resulting from a prolongation of lag phase in the presence of manganese (E. Eisenstadt, Ph.D. thesis, Washington

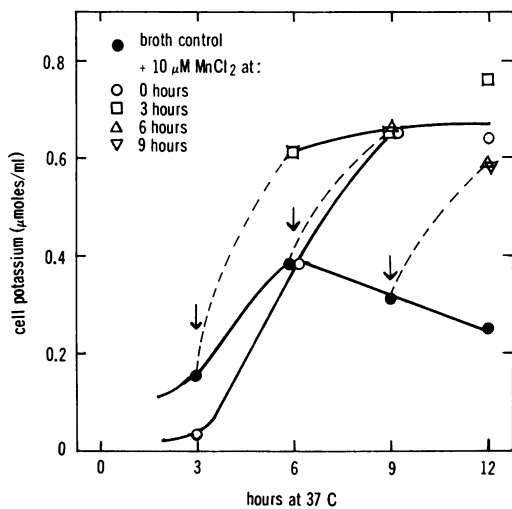


FIG. 2. Effect of varying the time of addition of manganese.  $MnCl_2$  ( $10 \mu M$ ) was added either at the time of dilution of the overnight culture or 3, 6, or 9 hr later. The lower value at 3 hr for the culture containing manganese does not represent a lower potassium content (per mg of dry weight) for the cells but, rather, results from a manganese-induced delay in the onset of exponential growth.

Univ., St. Louis, Mo., 1971). The cultures to which manganese was added at 3, 6, or 9 hr of incubation produced about half as many spores as the culture which had manganese throughout.

**Potassium flux experiments.** Because of the striking effect of manganese on the net potassium content of stationary-phase cells, the effect of manganese on the rate of  $^{42}K$  accumulation in sporulating cells and stationary phase-cells was examined (Fig. 3). After 12 hr of growth in unsupplemented broth, the rate of  $^{42}K$  accumulation was lower than the rate in sporulating cells (Fig. 3) or exponentially growing cells (data not shown; E. Eisenstadt, Ph.D. thesis, Washington Univ., St. Louis, Mo., 1971). When  $10 \mu M Mn^{2+}$  was added just prior to the addition of  $^{42}K$  at zero time, there was a marked stimulation of the initial rate of uptake which we know (Fig. 2) leads to a net increase in cellular potassium and ultimately to sporulation. The presence of  $100 \mu g$  of chloramphenicol per ml 2 min prior to the addition of manganese does not prevent the stimulation of potassium uptake (data not shown).

The effect of  $10 \mu M Mn^{2+}$  on protein synthesis in stationary-phase cells is seen in Fig. 4. Protein synthesis measured by the incorporation of  $^{14}C$ -leucine into trichloroacetic acid-insoluble material is stimulated after the ad-

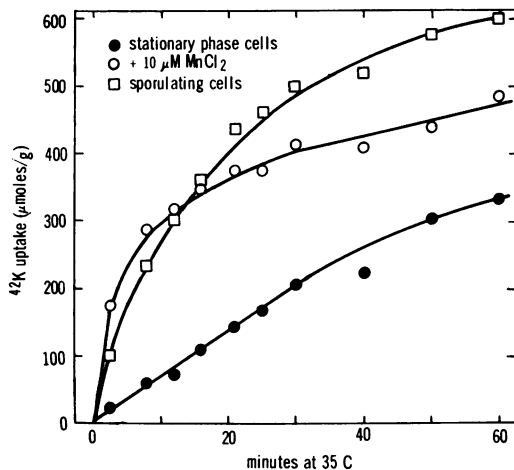


FIG. 3.  $^{42}K$  uptake by 12-hr sporulating or stationary-phase cells. *Bacillus subtilis* grown for 12 hr in tryptone broth (stationary phase) or tryptone broth plus  $10 \mu M MnCl_2$  (sporulating cells) were distributed into small flasks in a  $35 C$  shaking water bath.  $MnCl_2$  ( $10 \mu M$ ) was added to one flask with stationary-phase cells 30 sec before the addition of  $0.75 \mu Ci$  of  $^{42}K$  per ml. Samples (0.2 ml) were filtered and washed twice with 5 ml of tryptone broth.

dition of manganese to stationary-phase cells. The stimulation could be either direct or indirect as a result of the increased potassium content (5).

In contrast to spore calcium (3), the cellular potassium in sporulating cells is not firmly bound. With both exponential-phase and sporulating cells grown in the presence of  $^{42}K$ , 95% of the potassium is released by treatment with 1% toluene in the absence of visible lysis or loss of macromolecules (data not shown; E. Eisenstadt, Ph.D. thesis, Washington Univ., St. Louis, Mo., 1971). This treatment releases only 7% of the cellular calcium (3).

## DISCUSSION

Because sporulating cells are actively engaged in macromolecular synthesis (7) and because potassium is required for protein synthesis, it was not surprising to find that the intracellular potassium levels in exponentially growing cells and sporulating cells are equally high. The relationship between the manganese requirement for sporulation and potassium accumulation was, however, unexpected.

The mechanism of stimulation of potassium uptake by manganese is not understood. There may be a general stimulation of all membrane transport systems: magnesium accumulation by stationary-phase cells is also stimulated by addition of manganese (*manuscript in*

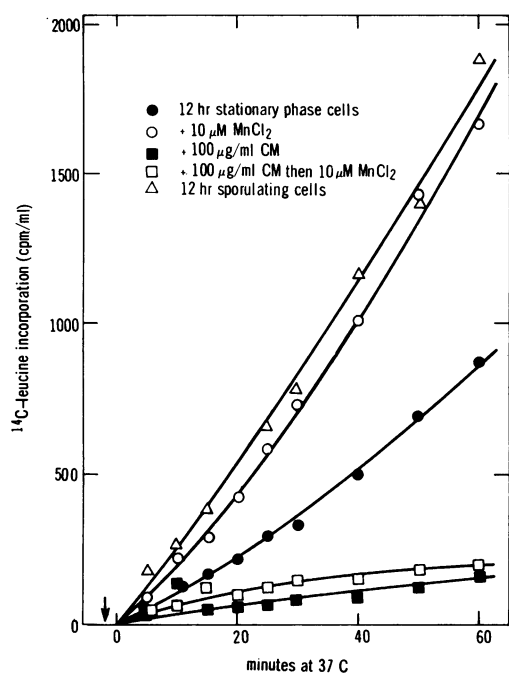


FIG. 4. Stimulation of protein synthesis by manganese. At 37 C, 12-hr stationary-phase and sporulating cells were distributed into flasks in a shaking water bath. Chloramphenicol or  $MnCl_2$ , or both, were added 2 min (at arrow) prior to the addition of 0.4  $\mu Ci$  of  $^{14}C$ -leucine per ml. Samples (0.2 ml) were pipetted into 2 ml of cold, 5% trichloroacetic acid. After 3 hr on ice, the samples were filtered and washed three times with 5 ml of cold, 1% trichloroacetic acid, and the dried filters were counted in a gas-flow counter.

preparation), but the effect of manganese on other transport systems (e.g., amino acids or sugars) was not examined.

These findings raise many questions about the function of manganese in the sporulation process and about the changes in bacterial manganese content during growth and sporulation. Although the mechanism of manganese stimulation of potassium uptake is not clear, it is known that stationary-phase cells such as those in Fig. 3 accumulate 100% of the 10  $\mu M$   $Mn^{2+}$  within 5 min of addition at 37 C (E. Eisenstadt, Ph.D. thesis, Washington Univ., St. Louis, Mo., 1971). Whatever the role(s) of manganese in sporulation (1) and in secondary metabolism (11) may be, it is probably not limited to regulating the potassium content of bacteria. Supplementing tryptone broth with

high concentrations of potassium (up to 100 mM) does not eliminate the manganese requirement for sporulation. No effect of 10  $\mu M$  manganese on exponential-phase potassium content has been seen.

#### ACKNOWLEDGMENTS

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