Regulation of Manganese Accumulation and Exchange in *Bacillus subtilis* W23

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An overnight culture of *Bacillus subtilis* W23 in low-manganese tryptone broth is unable to sporulate and becomes hyperactive with regard to the manganese active transport system during stationary phase. When manganese is added to cells in spent or fresh medium, the cells immediately accumulate a high proportion of the manganese available in the medium. When the hyperactive cells are diluted into broth containing 10 μM Mn²⁺, high intracellular manganese levels are reached, and inhibition of ribonucleic acid and protein synthesis occurs. This inhibition is relieved when the intracellular manganese concentration declines to the nontoxic levels characteristic of cells growing in 10 μM Mn²⁺. The release of the accumulated manganese is achieved by a reduction in the uptake rate for manganese while the efflux rate remains essentially constant. Inhibitors of ribonucleic acid and protein synthesis prevent the reduction of the high rate of manganese uptake and, therefore, high net concentrations of manganese are maintained in the presence of these inhibitors. The hyperactive manganese uptake system is temperature dependent and inhibited by cyanide and m-chlorophenyl carbonylcyanide hydrazone.

In the preceding paper (7) we have described the basic properties of the manganese active transport system of *Bacillus subtilis*. One of our findings was that manganese-starved stationary-phase *B. subtilis* W23 become "hyperactive" for manganese transport and that, when these cells are exposed again to manganese, they rapidly accumulate high levels of manganese. The cells subsequently release back into the medium up to 90% of the accumulated manganese (7) prior to the beginning of growth. In this paper we report studies on the mechanism of the release of manganese and on the regulatory conversion of the hyperactive manganese transport system into the system characteristic of growing cells.

**MATERIALS AND METHODS**

The conditions for growth and study of *Bacillus subtilis* W23 have been described (5-7) as have the methods for measurement of manganese transport and intracellular manganese content (7, 11). Actinomycin D was purchased from Sigma Chemical Co., St. Louis; m-chlorophenyl carbonylcyanide hydrazone (CCCP) from Calbiochem, Los Angeles; rifamycin SV sodium salt from Mann Research Lab., New York; and sodium cyanide from Fisher Scientific Co., Fair Lawn, N.J. Chloramphenicol was a gift from Parke, Davis & Co., Detroit. ¹C-leucine, ¹C-uracil, ⁴Rb and ⁴Mn were purchased from New England Nuclear Corp., Boston; and ⁴Mg from Brookhaven National Laboratory, Upton, N.Y. ⁴Mn was counted by liquid scintillation spectroscopy (7, 11), and ⁴Mg and ⁴Rb in water were counted via Cerenkov radiation (1, 6).

Overnight cultures hyperactive for manganese transport were produced by inoculation from a slant into tryptone broth supplemented with 25 mM K⁺, 1 mM Ca²⁺, and 1 mM Mg²⁺ ("supplemented broth," reference 7). The cultures were grown on a shaker at 37°C for 12 to 15 h and then diluted 20-fold into fresh media containing up to 10 μM Mn²⁺ for study.

Electron spin resonance (ESR) spectra were obtained at 20°C using a recording X-band spectrophotometer previously described (2), but modified with automatic frequency control. The first derivative of the absorbed microwave power was recorded as a function of the applied magnetic field. Aqueous solutions were placed in a cuvette with a 1-mm internal diameter. The modulation frequency was 100 kHz.

**RESULTS**

The time course of the rapid accumulation and release of manganese is shown in Fig. 1.
transport system distinct from the system responsible for manganese accumulation (6).

Energy dependence of manganese accumulation. If the hyper-accumulation of manganese by the "manganese-starved" overnight culture occurs via an active transport system, then it should be temperature dependent and sensitive to inhibitors of energy metabolism. The rate of manganese accumulation by an overnight culture of *B. subtilis* W23 was reduced by more than 95% at 4°C when compared with accumulation at 37°C (Fig. 2A). Similarly 10 mM sodium cyanide and 0.1 mM CCCP were inhibitory to the initial accumulation of manganese (Fig. 2A). Trace $^{54}$Mn (7) was added in the experiments in Fig. 2A and B, and the bacteria accumulated 75% of the radioactivity during about 5 min at 37°C.

Although the results in Fig. 2A show that the rapid accumulation of manganese is energy-dependent, as is manganese accumulation in growing *B. subtilis* (7), there are significant differences: 10 mM sodium azide, which inhibited $^{54}$Mn accumulation completely in log-phase *B. subtilis*, was essentially without effect on the hyperactive stationary-phase cells (Fig. 2B). $^{65}$Rb accumulation is also azide sensitive in vegetative cells but azide resistant in manganese-starved stationary-phase *B. subtilis* (Fig. 2C). Therefore, we assume that the azide resistance has its basis in differences in the electron transport systems between vegetative and stationary-phase cells. Such differences are known to occur between vegetative cells, sporulating cells, and spores (3, 8), and we plan to pursue this matter further.

Inhibition of RNA and protein synthesis. The dilution of a culture hyperactive for manganese transport into broth containing $10^{-8}$ M Mn$^{2+}$ resulted in an additional growth lag of almost 2 h as compared to the growth of the same culture diluted into broth containing 2 x $10^{-8}$ M Mn$^{2+}$ (Fig. 3A). In this experiment, more than 50% of the available manganese was rapidly accumulated at both the high and low manganese concentrations and then most of the accumulated manganese was released starting after 10 min with 2 x $10^{-8}$ M Mn$^{2+}$ and after 60 min with $10^{-5}$ M Mn$^{2+}$. At both manganese concentrations uracil and leucine incorporation began immediately at low levels during the "lag" phase after dilution of the overnight culture (Fig. 3B and C). Incorporation of both uracil and leucine was inhibited by the presence of $10^{-8}$ M Mn$^{2+}$. When the relative rates of ribonucleic acid (RNA) synthesis in the cells are plotted (Fig. 3C), one can see that there is a relatively low rate in the cells at 2 x
10^{-8} M Mn^{2+} during the first hour of incubation and that the rate of RNA synthesis increased dramatically as growth began (Fig. 3C). The initial rate of RNA synthesis was inhibited about 80% by the presence of 10^{-5} M Mn^{2+} in the culture (Fig. 3C), and this inhibition was relieved between 60 and 150 min so that the rate of RNA synthesis just prior to the onset of growth was about the same as that for the uninhibited (2 x 10^{-8} M Mn^{2+}) cells. With the inhibition of RNA synthesis, it is not surprising that the initial rate of protein synthesis was also inhibited (data not shown). The high and low manganese media differed in manganese content by a factor of 50; the hyperactive cells in low manganese accumulated about 0.2 µmol per g (dry weight; three times the concentration accumulated by cells growing in low-manganese broth [7]). The cells diluted into 10^{-5} M Mn^{2+} broth accumulated 100 µmol/g (dry weight) which is nearly 100 times the concentration of manganese in cells growing in 10^{-6} M Mn^{2+}-supplemented broth. Growth in 10^{-5} M Mn^{2+} began only when the cellular manganese had been reduced below 5 µmol per g (dry weight). The length of the growth lag induced by 10^{-5} M Mn^{2+} was variable from experiment to experiment (Fig. 1, 3 and 4); to some extent the higher the fraction of 10^{-8} M Mn^{2+} that was accumulated, the longer the lag (compare Fig. 1 and 3). We have not studied the basis for this variability but it probably is at least partially due to use of an "overnight" culture rather than cells in a more reproducible state.

Mechanism of release of the initially accumulated high manganese concentrations. Figures 1 and 3 showed examples of the phenomenon of the rapid accumulation of high concentrations of manganese followed by a period of rapid net manganese loss from the cells. The two most readily imagined mechanisms for this net loss phase would be either to reduce the accumulation system and continue the rate of exit or efflux of manganese from the cells at a high rate or to continue the rapid accumulation of manganese and increase strikingly the rate of efflux. Either of these or some combination of reduced uptake and increased efflux could account for the net loss. In the following experiments, we determined that the rate of manganese uptake is reduced approximately 90% (but not to zero) and that the rate of manganese efflux is relatively constant throughout the process. Figure 4 shows the results from one of these experiments where manganese exchange (efflux) was measured after diluting cells from radioactive into nonradioactive broth containing 10 µM Mn^{2+}. The time required for exchange of 50% of the manganese was about 10 min throughout the cycle of accumulation followed by net loss. There was no reproducible change in this "half-life" in the experiment in Fig. 4 or in additional similar experiments. Note that during the net loss phase the rate of manganese exchange plus loss (closed circles) was essentially equal to the rate of net loss (open circles), suggesting that there must be essentially no uptake of 4Mn. This was tested directly in the next experiment where cells were allowed to accumulate nonradioactive manganese under the same conditions used in the experiment in Fig. 4 and then periodically diluted into 10 µM ^{46}Mn, and the initial rate of manganese accumulation was determined (Fig. 5). ^{46}Mn uptake was essentially the same at 2, 10, 20, 30 and 40 min; the rate was considerably less at 60 min, and the rate of manganese accumulation at 90 or 120 min was essentially zero. Taken together, the experiments in Fig. 4 and 5 establish that the net loss phase reflects a reduction in the rate of manganese accumulation without a significant change in the rate of manganese
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Fig. 3. Manganese accumulation and inhibition of RNA and protein synthesis. Duplicate 20-fold dilutions were made from an overnight culture into flasks containing tryptone broth or broth plus 10 μM Mn⁺⁺ and either ⁴⁴Mn, ¹⁴C-leucine, or ¹⁴C-uracil (0.1 μCi/ml). Growth was followed in Klett side-arm flasks (A). ⁴⁴Mn accumulation was determined by filtering and washing 1.0-ml samples at various times (A). Incorporation of ¹⁴C-uracil into RNA and ¹⁴C-leucine into protein was determined by trichloracetic acid precipitation followed by filtration (B). The rates of RNA synthesis (C) were calculated from the data in Fig. 3B and from short-term labeling data averaging over 20- to 30-min periods.

exit.

Requirement for RNA and protein synthesis. One can ask whether either phase, the initial rapid accumulation or the phase of net loss, depends on RNA or protein synthesis after dilution of the overnight B. subtilis culture. When added to the medium before dilution of the overnight culture, chloramphenicol (blocking protein synthesis) and actinomycin D (blocking RNA synthesis) did not affect the initial accumulation of manganese (Fig. 6). When added at 13 min, at the peak of the accumulation phase, the antibiotics completely prevented the subsequent net loss of manganese. When added at 28 min, about one-fifth through the net loss phase, ⁴⁴Mn net loss stopped and the cells maintained a manganese content at the level representative of the time of addition of chloramphenicol or actinomycin D. When added at 58 or 88 min, after the loss of manganese, actinomycin D or chloramphenicol was without much effect on cellular manganese content (Fig. 6). Thus the initial phase of rapid accumulation of ⁴⁴Mn does not depend on concomitant RNA or protein synthesis, but the phase of net loss requires continuous RNA and (or) protein synthesis.

RNA and (or) protein synthesis is required to turn off (or down) the rate of manganese accumulation. This is seen in Fig. 7 using another inhibitor of RNA synthesis, rifamycin SV. Rifamycin, when present in the fresh medium at the time of dilution, did not affect the initial accumulation of 1.0 μM ⁴⁴Mn; rifamycin did prevent the subsequent phase of net manganese loss, which we have shown is due to a reduction in the rate of accumulation of manganese. This point is seen directly in the experiment in Fig. 7, where samples were diluted into fresh nonradioactive medium containing rifamycin but not ⁴⁴Mn, at time zero, and then diluted further into ⁴⁴Mn at 2, 10, 20,
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but rather is turned down.

The rate of manganese accumulation increased again by 90 and 120 min as the cells began to grow (Fig. 7; see below). The cycle of accumulation followed by net loss was complete in 75 min in the experiment in Fig. 7 but required more than 120 min in the experiments in Figs. 3 and 4, primarily due to the inhibition by the higher Mn2+ concentration used (10 μM versus 1.0 μM). Over 2 h in the experiment in Fig. 7, there was a slight net loss of manganese and a slight decrease in the rate of manganese accumulation in the presence of rifamycin. We do not know whether this was due to a “leakiness” in the rifamycin effect on the manganese transport system or just due to the overall toxicity of rifamycin and (or) high manganese upon more general cellular metabolism.

In Fig. 8A we see that inhibition of RNA and protein synthesis with rifamycin at the time of “peak” manganese accumulation resulted in retention of high cellular manganese and that the addition of an inhibitor of RNA synthesis at any time during the net loss phase brought about a rapid cessation in manganese loss. The cells either retained the level of manganese

30, 40, 60, 90 or 110 min. The cells that had been incubated in rifamycin retained a high rate of manganese accumulation, whereas the cells without rifamycin had an accumulation rate of 13 nmol/min at 2 and 10 min which decreased to 10 nmol/min at 20 min, 7 nmol/min at 40 min, and finally to a low of 1 nmol/min at 60 min, when 90% of the net manganese (Fig. 7, open circles) had been released. The rate of accumulation of 54Mn never decreased completely to “0” in the experiment in Fig. 7. Therefore it appears that the manganase transport system is not turned off
from time of addition of rifamycin or accumulated somewhat higher levels (Fig. 8A).

Continuous RNA and protein synthesis was required during the net loss phase. The separate experiment in Fig. 8B shows two additional aspects of the cycle of manganese accumulation and loss. First, when net Mn$^{2+}$ accumulation is normalized for growth and we measure Mn$^{2+}$ per gram of cell mass (open circles, Fig. 8B), we see that the cellular manganese remained essentially constant beyond incubation for 60 min and that the secondary increase in cellular manganese in Fig. 8A and earlier figures was due to an increase in cell mass. The rate of manganese uptake per cell did not increase. This is seen more directly in the short-term labeling experiment in Fig. 8B. The rate of $^{54}$Mn accumulation had decreased to a minimum by 45 min, and, although the absolute rate increased from 80 through 130 min, the rate of manganese accumulation per unit of cell mass remained constant (Fig. 8B). When rifamycin was added to the cells and then $^{54}$Mn accumulation was measured 20 min later, the rate of manganese accumulation was either as high as it was at the time of addition of rifamycin or higher (Fig. 8B; compare the closed triangle data with open triangle data from 20 min earlier).

"State" of the accumulated manganese.

The state of the accumulated manganese was studied by adding 10 $\mu$M Mn$^{2+}$ directly to the overnight cultures. All of the 10 $\mu$M $^{54}$Mn was accumulated within 40 sec at 25 C (data not shown) but with a measured cell mass of 1.35 g (dry weight) per liter; this corresponds to only 5 $\mu$mol per g. $^{54}$Mn-loaded stationary-phase cells were converted to spheroplasts with lysozyme and retained 97% of the radioactivity, showing that the Mn$^{2+}$ was intracellular and not bound to the cell surface. When the spheroplasts were lysed osmotically, they released into the medium 75% of the $^{54}$Mn in a form not sedimented by centrifugation at 30,000 x g for 30 min; when the spheroplasts were lysed with detergent (0.1% sodium dodecyl sulfate), less than 5% of the $^{54}$Mn was sedimentable. Centrifugation of the 30,000 x g supernatant fluid from detergent-lysed spheroplasts for 3 h at 100,000 x g sedimented only 15% of the $^{54}$Mn in the 30,000 x g supernatant fluid. These experiments and the exchangeability of the accumulated $^{54}$Mn (e.g., Fig. 4) show that the intracellular manganese is not tightly bound in a precipitate. Nevertheless, the intracellular manganese is not completely free: comparison of the ESR signal of 10 $\mu$M Mn$^{2+}$ in tryptone broth (Fig. 9A) with the signal from an overnight B. subtilis culture to which 10 $\mu$M Mn$^{2+}$ had just been added (Fig. 9B) shows that the hyperfine structure spectrum characteristic of the free paramagnetic Mn$^{2+}$ ion in solution was entirely lost upon accumulation by the cells. Loss of the hyperfine structure ESR spectrum also occurs upon incorporation of manganese into spores (12), but manganese in spores is no longer exchangeable (7). Manganese accumulated by mitochondria shows a "quenched" ESR spectrum attributed to binding of the manganese to intramitochondrial
macromolecules (9).

Other bacteria. B. subtilis strain W23 was the subject of this investigation of regulation of manganese transport. Preliminary experiments with a B. subtilis 168 derivative (SMY of Schaeffer) showed a Mn²⁺-dependent growth delay similar to that we have observed with B. subtilis W23, suggesting that other B. subtilis strains can produce the hyperactive manganese system. However, Escherichia coli K-12 and B. cereus T, when grown into stationary phase in low manganese tryptone broth, retain manganese transport systems that show Michaelis-Menten kinetics but no increase in $V_{\text{max}}$ (data not shown).

DISCUSSION

The results in this paper describe a rather novel form of regulatory control of active transport in bacterial cells. B. subtilis W23 under conditions of manganese starvation develops a higher potential for manganese active transport. Upon exposure to manganese, the cells rapidly accumulate high levels of manganese via the hyperactive transport system. Then RNA and protein synthesis are required to reduce the rate of functioning of the manganese transport system to a level more characteristic of growing cells. The cells that have over-accumulated manganese release as much as 90% of the accumulated manganese.

Regulatory control of other bacterial transport systems has been reported but there are significant differences. Hochstadt-Ozer and Stadtman (10) found higher levels of the adenine uptake system in E. coli after maintaining the cells under conditions of adenine starvation. They did not study the process of return to the state characteristic of growing cells. Dreyfuss and Pardee (4) found a regulatory overshoot and net loss phase in the accumulation of sulfate in sulfur-starved cells of Salmonella typhimurium. Here, however, regulation did not involve the synthesis of proteins altering transport rates, since the rapid accumulation and net loss phases took less than 5 min at 37 C and were resistant to chloramphenicol.

We cannot be sure from the present experiments whether the manganese transport system is turned down or is turned off. In a population of cells exposed to 1.0 μM Mn²⁺ some residual manganese accumulation activity always remained at the low point following the net loss phase (Fig. 7 and 8B and additional experiments). This residual activity either reflects careful regulatory "modulation" or the role of the regulatory protein involves an enzymatic inactivation of the manganese transport system. Then new manganese transport systems would be synthesized to maintain the cellular level and during growth. The explanation for the residual activity at the low point would, as before, involve population heterogeneity; not all of the cells in a population would have their manganese transport systems inactivated at the same time. If the inactivator was unstable or was consumed during its action on the transport system, this would account for the need for continuous protein synthesis during the phase of net manganese loss.

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