Manganese Transport in *Brevibacterium ammoniagenes* ATCC 6872

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Uptake of manganese by *Brevibacterium ammoniagenes* ATCC 6872 was energy dependent and obeyed saturation kinetics ($K_m = 0.65$ μM; $V_{max} = 0.12$ μmol/min per g [dry weight]). Uptake showed optima at 27°C and pH 9.5. $^{54}$Mn$^{2+}$ accumulated by the cells was released by treatment with toluene or by exchange for unlabeled manganese ions, via an energy-dependent process. Co$^{2+}$, Fe$^{3+}$, Cd$^{2+}$, and Zn$^{2+}$ inhibited manganese uptake. Inhibition by Cd$^{2+}$ and Zn$^{2+}$ was competitive ($K_i = 0.15$ μM Cd$^{2+}$ and 1.2 μM Zn$^{2+}$). Experiments with $^{65}$Zn$^{2+}$ provided no evidence for Zn$^{2+}$ uptake via the Mn$^{2+}$ transport system.

Most organisms, possibly all, require manganese as a trace element (13). Among microorganisms, it is required as a cofactor for various enzymes such as the superoxide dismutase (EC 1.15.1.1) in *Escherichia coli* and the RNA polymerase (EC 2.7.7.6) in *Lactobacillus curvatus* and also for sporulation in bacilli (13). It also serves as an O$_2$ scavenger in *Lactobacillus plantarum* (1). Manganese can be taken up either as a low-affinity substrate of the Mg$^{2+}$ transport system or via a specific Mn$^{2+}$ transport system with high affinity (14). In this report, the term manganese transport designates uptake via the Mn$^{2+}$-specific system. Manganese transport systems have been described in a variety of bacteria, including *E. coli*, *Rhodospseudomonas capsulata*, *Staphylococcus aureus*, *L. plantarum*, *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus brevis* (1, 4, 7, 14). In all these organisms, either a growth requirement for the metal cannot be demonstrated or else the molecular basis for their manganese requirement is not entirely clear.

*Brevibacterium ammoniagenes* clearly requires manganese for growth. Depletion of the metal leads to selective inhibition of DNA synthesis and induces unbalanced filamentous growth similar to the "thymineless death" phenomenon in *E. coli* (3). Manganese dependence of the ribonucleotide reductase (EC 1.17.4), which is central to the synthesis of deoxyribonucleotides, was shown to be the molecular basis for the manganese requirement in *B. ammoniagenes* (2, 6, 11; J. Schmid, Ph.D. thesis, Universität Hannover, Hanover, Federal Republic of Germany, 1985). Because of the well-documented requirement of *B. ammoniagenes* for manganese and the extensive knowledge concerning its molecular basis, it was of interest to characterize manganese uptake by this organism.

*B. ammoniagenes* ATCC 6872 was grown at 27°C in dilute tryptone broth (12). Overnight cultures were diluted 6- to 11-fold with fresh medium and incubated on a gyratory incubator shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 230 rpm. When a cell density of $2.06 \times 10^8$ cells per ml was reached, $^{54}$Mn$^{2+}$ (0.4 to 3.7 kBq/ml) or $^{65}$Zn$^{2+}$ (6 to 37 kBq/ml) (Amersham Buchler GmbH & Co., Braunschweig, Federal Republic of Germany) was added to the mid-log-phase cells. Samples (1 ml) were filtered on membrane filters (0.2-μm pore size), rinsed twice with 5 ml of dilute tryptone broth at room temperature, and counted with a 12SW/3aW3 sodium iodide crystal equipped with an RCA multiplier (Harshaw Chemie B. V., De Meern, The Netherlands) and an LP 4840 Nokia Multichannel Pulse Analyzer (Nokia, Helsinki, Finland). Binding of $^{54}$Mn$^{2+}$ to filters and cell surfaces did not exceed 0.5% of the total radioactivity. This background was subtracted from the radioactivity of the cell samples. The dilute tryptone broth contained 0.2 μM Mn$^{2+}$ and 1.8 μM Zn$^{2+}$ (determined with a Perkin-Elmer atomic absorption spectrophotometer, using a flame assay mode [The Perkin-Elmer Corp., Norwalk, Conn.]). Due to uptake during the time between inoculation and the start of the transport experiments, the medium contained only 50 nM Mn$^{2+}$ and 1.5 μM Zn$^{2+}$ at the time the transport assays were undertaken (determined by radioisotope distribution).

Manganese uptake by *B. ammoniagenes* is an energy-dependent process that may be affected by inhibitors of energy metabolism. Carboxyl cyanide $m$-chlorophenylhydrazone (CCCP) (0.5 mM) inhibited uptake by 96%, 2,4-dinitrophenol (0.5 mM) inhibited uptake by 50%, and cyanide (1 mM) inhibited uptake by only 15%. Because proton conductors strongly inhibit the manganese transport system of *B. ammoniagenes*, it may be driven by the electrochemical potential of protons, as is also thought to be true for the manganese transport system of *E. coli* (13) and *L. plantarum* (1).

Acquisition of manganese by *B. ammoniagenes* is very efficient. Assuming a cell water content of 3 ml/g (dry weight) (Schmid, Ph.D. thesis) and taking into account only that part of the manganese that is readily exchangeable (see below), the internal manganese concentration reaches approximately 0.2 mM when the external manganese concentration is 50 nM, i.e., a concentration gradient of 4,000:1.

Manganese uptake by *B. ammoniagenes* occurs over a wide range of temperature and pH (Fig. 1). The optimal pH for manganese uptake was 9.5. However, the shape of the curve suggested a second optimum around pH 7.5. We assume from growth experiments that the optimum at pH 9.5 is caused by maximal metabolic activity of the cells, whereas a pH around 7.5 is optimal for the function of the Mn$^{2+}$ carrier molecule.

Accumulation of manganese obeyed saturation kinetics. Lineweaver-Burk plots of initial uptake at pH 7.2, the pH of tryptone broth, were obtained over a concentration range from 0.05 to 2.0 μM Mn$^{2+}$. The $K_m$ was 0.65 ± 0.34 μM, and the $V_{max}$ was 7.2 ± 2.4 pmol/min per 10$^8$ cells (0.120 ± 0.04 pmol/min per 10$^8$ cells).
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Temperature (°C)

\[ \text{Uptake (pmol/10^6 Cells)} \]

\[ \begin{array}{cccccccc}
0 & 5 & 10 & 15 & 20 & 25 & 30 & 35 \\
5 & 3 & 2 & 1 & 0 & -1 & -2 & -3 \\
\end{array} \]

FIG. 1. Temperature and pH dependence of Mn\(^{2+}\) uptake. Cells were exposed to 50 nM \(^{54}\text{Mn}^{2+}\), and uptake was assessed after 10 min. Cells were preincubated at assay temperature for 5 min and at assay pH for 20 min. All pH trials (\(\ominus\)) were done at 27°C, and all temperature trials (\(\bigcirc\)) were done at pH 7.2. Background counts were determined under all conditions and subtracted from the radioactivity in cell samples.

µmol/min per g (dry weight) \((n = 3)\). We note that these assays were not done at the optimal pH for Mn\(^{2+}\) uptake and, as growth experiments showed, not under conditions where growth was limited by manganese.

\(^{54}\text{Mn}^{2+}\) accumulated by \(B.\ ammoniagenes\) could be released rapidly and in the absence of visible lysis by addition of 1% toluene or by the addition of nonradioactive manganese \((10 \mu M)\) (Fig. 2). The bulk of the accumulated manganese thus appears to exist in a relatively free state within the cells. The addition of the proton-conducting ionophore CCCP did not, in itself, lead to a significant loss of manganese from the cells, but it did inhibit the exchange of internal radioactive manganese for nonradioactive external manganese (Fig. 2).

To determine the specificity of the Mn\(^{2+}\) transport system, a variety of divalent cations was added. Mn\(^{2+}\) uptake was unaffected by a 20,000-fold excess of Mg\(^{2+}\), proving that the uptake of \(^{54}\text{Mn}^{2+}\) observed in this study was not caused by the activity of the Mg\(^{2+}\) transport system. When added at 1 or 2 µM, Co\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\), but not Ni\(^{2+}\) or Cu\(^{2+}\), inhibited uptake (data not shown). The inhibition of uptake by Cd\(^{2+}\) and Zn\(^{2+}\) was studied in further detail and found to be competitive, with \(K_s\) of 0.15 ± 0.02 µM Cd\(^{2+}\) \((n = 2)\) and 1.22 ± 0.15 µM Zn\(^{2+}\) \((n = 2)\) (Fig. 3). Although competitive inhibition of Mn\(^{2+}\) uptake by Co\(^{2+}\), Fe\(^{2+}\), and Cd\(^{2+}\) has been observed in other bacteria (1, 14), competitive inhibition of Mn\(^{2+}\) uptake by Zn\(^{2+}\) has apparently not been reported previously for procaryotes. There is still no direct evidence for bacterial Mn\(^{2+}\) transport systems despite various efforts to detect them (9, 14). Zn\(^{2+}\), like Mn\(^{2+}\), is required as a cofactor for several bacterial enzymes, but a nutritional requirement is rarely demonstrable (5).

Because Zn\(^{2+}\) competitively inhibits Mn\(^{2+}\) uptake and a Zn\(^{2+}\) requirement for growth has been demonstrated in \(B.\ ammoniagenes\) (8), we expected to find active Zn\(^{2+}\) uptake via the Mn\(^{2+}\) transport system. This expectation was, however, not realized in uptake studies with \(^{65}\text{Zn}^{2+}\) (data not shown). The rate of Zn\(^{2+}\) uptake was very low, about 1/10 of that observed for Mn\(^{2+}\), and at equilibrium only 1.5% of the radionuclide had been incorporated by the cells. \(^{65}\text{Zn}^{2+}\) uptake did not occur via the magnesium transport system since it was not inhibited by 1 mM Mg\(^{2+}\). Manganese (2 µM) did, however, also not inhibit \(^{65}\text{Zn}^{2+}\) accumulation. Zn\(^{2+}\) accumulation was not inhibited by CCCP and did not follow saturation kinetics.

The characteristics of the manganese transport system of \(B.\ ammoniagenes\) are comparable to those described for other procaryotes, except for its competitive inhibition by Zn\(^{2+}\). Our failure to demonstrate active zinc uptake may be caused by the low zinc requirements of bacteria (5), by the relatively high Zn\(^{2+}\) concentration of the tryptone broth, and possibly by an extremely high affinity of the transport system for zinc (14).

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LITERATURE CITED