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EFFECTS OF Mg^{2+} AND Ca^{2+} ON Fe^{2+} UPTAKE BY *BIFIDOBACTERIUM THERMOPHILUM*

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Abstract—Ferrous iron uptake was investigated in *Bifidobacterium thermophilum* (*B. thermophilum*) in the presence of Mg^{2+} and Ca^{2+} with the following findings:

1. Mg^{2+} inhibited Fe^{2+} accumulation in the cells in a dose-dependent manner at 37°, but not at 0°. Removal of Mg^{2+} from the medium resulted in a resumption of rapid iron uptake.
2. Mg^{2+} had no effect on the binding of Fe^{2+} by *B. thermophilum* protoplasts, its cellular particulate fraction, or distribution between the particulate and soluble fractions.
3. Ca^{2+} exerted a stimulatory effect on iron uptake by *B. thermophilum*, but was not able to reverse the inhibitory effects of Mg^{2+}.
4. It was concluded that Mg^{2+} has no effect on the binding of iron on the surface or interior of *B. thermophilum* and that it affected the Fe^{2+} transport mechanism (permease) in a reversible manner. It is possible that iron and magnesium share the same permease in this microorganism.

INTRODUCTION

Bifidobacteria are microaerophilic anaerobes that inhabit the gastrointestinal tracts of human beings and animals, and are believed to contribute to the well-being of both infants and adults (Bezkorovainy and Miller-Catchpole, 1989). For that reason, bifidobacteria have been widely used as supplements with various types of dairy products (Kroger *et al.*, 1989). *Bifidobacterium thermophilum* (*B. thermophilum*) is a rumen organism, which readily forms protoplasts and whose iron metabolism has been investigated to some extent in our laboratory (Kot and Bezkorovainy, 1991; Kot and Bezkorovainy, 1993; Kot *et al.*, 1993). It serves as a convenient prototype for other bifidobacterial species as well.

Ferrous iron uptake by bifidobacteria is energy-dependent, and these organisms are capable of accumulating large amounts of iron providing that they had been grown in a metal-poor environment (Kot and Bezkorovainy, 1991). Uptake of iron, carried out in a simple inorganic buffer containing a carbon source such as lactose or glucose, is inhibited by a variety of metal ions (Bezkorovainy *et al.*, 1987), which includes Mg^{2+} but not Ca^{2+} (Bezkorovainy *et al.*, 1987; Bezkorovainy and Solberg, 1989). The latter phenomenon has been somewhat of a puzzle in view of the ubiquity of Mg^{2+} and its essentiality in practically all life forms. This report explores in some detail, the effects of Mg^{2+} and Ca^{2+} on iron uptake by *B. thermophilum*.

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MATERIALS AND METHODS

Microorganisms and their fractionation

B. thermophilum (ATCC 25866) was purchased from American Type Culture Collection (Rockville, MD). It was grown in the trypticase-phytone-yeast extract (TPY) medium (Scardovi, 1986), from which metals had been omitted. This medium has been termed the "modified TPY medium" (Kot and Bezkorovainy, 1991). The culture was routinely examined microscopically using the Gram stain (bifidobacteria are Gram-positive) and for the presence of phospho-fructoketolase enzyme, which is unique to bifidobacteria (Scardovi, 1986). A pellet of organisms is identified as that amount of bifidobacteria, which is present in a 5 ml suspension with an $A_{610} = 1.2$. Such a suspension contains 8.5×10^7 to 1×10^8 organisms per ml (Kot *et al.*, 1993).

Protoplasts of *B. thermophilum* were prepared by lysozyme and protease treatments as described previously (Kot *et al.*, 1993). *B. thermophilum* cells were disrupted by sonication and separated into the particulate and soluble fractions by centrifugation as previously described (Kot and Bezkorovainy, 1993).

Analytical procedures

Protein determinations were done by the method of Lowry *et al.* (1951). Lactic acid was determined with lactate dehydrogenase using kits supplied by Sigma Corporation (St. Louis, MO). Radioactivity was measured in a Gamma-4000 gamma counter (Beckman Instruments, Palo Alto, CA).

Iron uptake measurements

$^{59}Fe^{2+}$ was used for iron uptake studies by cell suspensions ($A_{610} = 1.2$) in the modified Hanks solvent (Kot and Bezkorovainy, 1991). This solvent had the following ingredients: 0.4 g KCl, 8 g NaCl, 0.14 g $CaCl_2$, 8.2 g sodium acetate

and 2 g glucose/l adjusted to pH 5.0 with HCl. In some instances, wherever stated, the $CaCl_2$ was omitted. Washing of cells was done with 0.2 M acetate buffer at pH 5.0 at 0°C.

Incubations of cells with $^{59}Fe^{2+}$ were done in air at 0° or 37°C for varying periods of time, usually 1 hr. Lactate was measured to ascertain cell viability. For the removal of cell surface $^{59}Fe^{2+}$, the cells were washed for 30 min at 0°C with the modified Hanks solution containing 2 mM non-radioactive $FeSO_4$.

Materials

Peptone and trypticase used for the TPY medium preparation were purchased from BBL Microbiology Systems (Cockeysville, MD). $^{59}Fe^{2+}$ was obtained from DuPont Laboratories (Boston, MA). All other chemicals came from the Sigma Corporation (St. Louis, MO).

RESULTS

Effect of Mg^{2+} on $^{59}Fe^{2+}$ uptake by B. thermophilum

Iron uptake by the cells was measured at various iron concentrations (23–200 μM) in the presence of increasing Mg^{2+} concentrations. Typical data are given in Fig. 1. It is seen that there is a dose dependence in the action of Mg^{2+} Another phenomenon of note is the fact that in the iron concentration range of 23–100 μM, at high [Mg^{2+}], the amount of iron associated with the cells reached a maximum at 10 min and then declined over a period of time. Such samples, however, produced increasing amounts of lactate, hence such a decline cannot be ascribed to cell lysis. This behavior was not observed when iron concentration in the suspension medium was near 200 μM. In the latter case, in the presence of 300 μM Mg^{2+} iron uptake at the 60 min point was only 10%that of the control [Fig. 1(B)].

Iron uptake by *B. thermophilum* at 0° in the presence of Mg^{2+} is shown in Table 1. This table indicates that iron uptake is biphasic, as was previously reported (Kot and Bezkorovainy, 1993), however, Mg^{2+} had absolutely no effect on iron binding by the cells.

Figure 2 shows the effect of Mg^{2+} removal from the iron uptake medium one hour after starting the iron uptake experiment. It is seen that this causes a resumption of iron uptake (Fig. 2, curve B). Lactate production continued throughout the 2 hr experimental time-frame.

Distribution of iron in cell components in the presence of Mg^{2+}

B. thermophilum cells were incubated with iron in the presence of Mg^{2+}, surface-bound iron was washed off, and cells were then disrupted by sonication. $^{59}Fe^{2+}$ was then measured in the particulate and soluble fractions, as was described previously (Kot and Bezkorovainy, 1993). The purpose of this experiment was to determine if Mg^{2+} serves to alter iron binding to the cytosol portion of the membrane. The results are shown in Fig. 3. The two resulting curves were compared using the unpaired *t*-test. The one-tailed *P*-value was 0.0690, indicating a marginally significant difference. Correlation coefficients for curves A and B were 0.77 and 0.89 respectively. Thus, Mg^{2+} had a marginally positive effect on the binding of iron by the insoluble components of the cellular interior.

In a separate experiment, cells that had not been preloaded with iron were disrupted by sonication, and their particulate fraction used for iron-binding studies. Previously, such results, performed at 0 or 37°C, reflected surface binding of iron by bifidobacteria (Kot and Bezkorovainy, 1993). At 0°C, there was no significant difference in the binding of $^{59}Fe^{2+}$ by

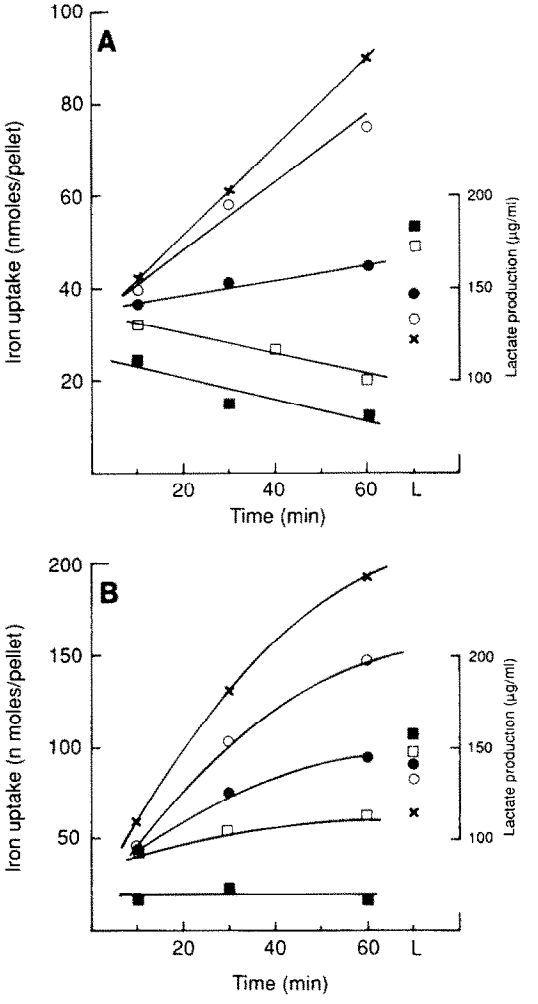

Fig. 1. Effect of Mg^{2+} on $^{59}Fe^{2+}$ uptake by *B. thermophilum* at 37°C as a function of time. (A) represents 103 ± 4.50 μM $^{59}Fe^{2+}$ in the presence of the following Mg^{2+} concentrations (μM): 50 (○), 100 (●), 150 (□) and 200 (■). L on the *x*-axis represents lactate production after 60 min of incubation. (B) represents results at 190 ± 6.82 μM $^{59}Fe^{2+}$ in the presence of the following Mg^{2+} concentrations (μM): 100 (○), 150 (●), 200 (□) and 300 (■). L has the same meaning as in part A. In both parts A and B, × represents control conditions (no Mg^{2+}).

Table 1. Uptake of $^{59}Fe^{2+}$ by *B. thermophilum* at 0°C in the presence and absence of Mg^{2+} (in nmol/pellet)

		Time (min)			
$[^{59}Fe^{2+}]$ (μM)	$[Mg^{2+}]$ (μM)	10	20	30	60
48.0	0	2.20	2.55	3.20	5.19
50.0	200	2.42	2.44	2.86	5.32
98.0	200	5.69	5.84	6.11	9.68
100	0	5.48	5.73	6.62	10.5
185	200	9.96	—	14.3	19.1
210	400	8.85	—	14.1	18.3
208	600	9.53	—	14.0	18.4

Table 2. Effect of Mg^{2+} on the binding of $^{59}Fe^{2+}$ by the particulate fraction of *B. thermophilum* at 0°C as a function of time (in nmol/mg protein)

		Time (min)		
$[^{59}Fe^{2+}]$ (μM)	$[Mg^{2+}]$ (μM)	10	30	60
53.0	0	1.50	2.09	3.08
48.0	200	1.32	1.86	2.51
105	0	1.78	3.69	4.12
97.0	200	1.45	2.82	4.04

the control and in the presence of various concentrations of Mg^{2+} (Table 2).

Displacement of $^{59}Fe^{2+}$ by Mg^{2+} from iron-preloaded cells

B. thermophilum cells were preloaded with iron, then suspended in an iron-free modified Hanks medium in the presence of various Mg^{2+} concentrations. The results are shown in Table 3. It may be observed that Mg^{2+} exerted only a minor effect on the loss of iron by the cells, and that the maximum Mg^{2+} effect was observed at about 400 μM concentration.

Binding of $^{59}Fe^{2+}$ by B. thermophilum protoplasts

It had been previously shown that *B. thermophilum* protoplasts could bind $^{59}Fe^{2+}$ at their surfaces only and that this binding is almost instantaneous. Moreover, the extent of iron binding was the same at both 0° and 37°C and no iron was internalized (Kot *et al.*, 1993). *B. thermophilum* protoplasts were incubated at 0°C for 10 min with 207 ± 9.43 μM $^{59}Fe^{2+}$ in the presence of 200–600 μM Mg^{2+} and iron binding was then measured. No difference between the control and the Mg^{2+} treated cells was observed. Binding of iron was 73.9 ± 2.43 nmol/pellet at all Mg^{2+} concentrations, whereas the binding in the control was 77.0 nmol/pellet.

Effect of Ca^{2+} on $^{59}Fe^{2+}$ uptake by B. thermophilum

Various concentrations of Ca^{2+} were tested for their effects on iron uptake by *B. thermophilum* at 37°C. Ca^{2+} alone had a mild stimulatory effect on $^{59}Fe^{2+}$ uptake, and this stimulation was dose-dependent. This was observed at several iron concentrations, though only one range (130–139 μM) is shown in Table 4. Ca^{2+} was not able to abolish or even moderate the inhibitory effect of Mg^{2+} at 3 mM concentration. If anything, it potentiated the effect of Mg^{2+} (Table 4).

DISCUSSION

There is little doubt that Mg^{2+} but not Ca^{2+} has a profound inhibitory effect on the uptake of Fe^{2+} by *B. thermophilum*, and by extension, in all bifidobacteria. If we divide the iron uptake phenomenon

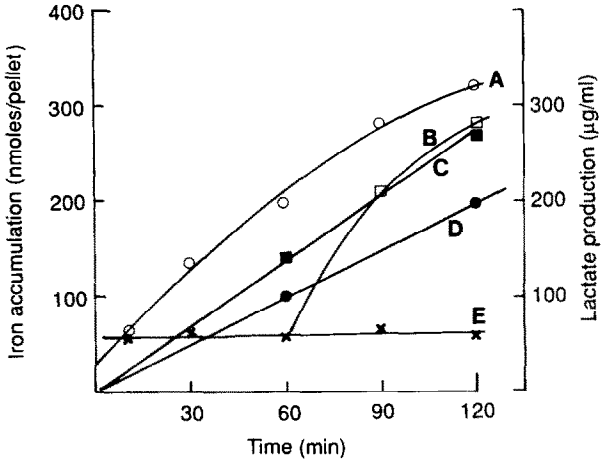

Fig. 2. Iron uptake and lactate production by *B. thermophilum* cells at 37°C in the presence and absence of 200 μM Mg^{2+} and $[^{59}Fe^{2+}] = 212 \pm 24.0$ μM. Curve (A) (○) represents the normal control: cells were incubated with iron for 60 min, then centrifuged, washed and reincubated with the same iron concentration. Curve E (×) represents cells that were incubated with iron and Mg^{2+} for 60 min, then centrifuged, washed and reincubated with the same iron and Mg^{2+} concentrations. Curve B (□) represents cells in curve E, however, after centrifugation and washing after 60 min, these cells were re-incubated with iron in the absence of Mg^{2+}. Lactic acid production is shown by curves C and D: curve D (●) was generated under conditions of curve A, and curve C was obtained under conditions established for both curves B and E.

Table 3. Displacement of $^{59}Fe^{2+}$ from iron-preloaded cells by various Mg^{2+} concentrations. Cells were preloaded with $^{59}Fe^{2+}$ for 60 min at 37°C at $229 \pm 7.55\ \mu M$ iron concentration, then suspended in an iron-free medium in the absence (control) and presence of Mg^{2+}

Total $^{59}Fe^{2+}$ taken up (nmol/pellet)	$[Mg^{2+}]$ (μM)	Iron remaining in cells (in % of that taken up)	
		30 min	60 min*
210	0	77	65
214	200	74	61
190	400	69	55
206	600	70	56

*Incubation time in iron-free medium. Iron not recovered in the cells was found in the incubation medium.

Table 4. The effects of Ca^{2+} on the uptake of $^{59}Fe^{2+}$ by *B. thermophilum* in the presence and absence of Mg^{2+} (in nmol/pellet)

			$^{59}Fe^{2+}$ uptake		
$[^{59}Fe^{2+}]$ (μM)	$[Ca^{2+}]$ (mM)	$[Mg^{2+}]$ (μM)	10 min	30 min	60 min
130	0	0	33.0	52.9	71.8
134	1.26*	0	38.4	64.4	86.2
139	2.26	0	47.4	85.2	117
130	2.76	0	49.4	89.0	112
106	0	0	24.0	40.5	55.6
107	3.00†	0	49.7	79.0	105
119	0	100	25.4	35.9	35.9
104	3.00	100	24.9	27.5	27.4
107	0	200	14.2	13.8	11.7
103	3.00	200	8.48	7.94	7.63

*This is the normal modified Hanks solution used in this work.
†Ca^{2+} precipitated beyond the 3 mM concentration.

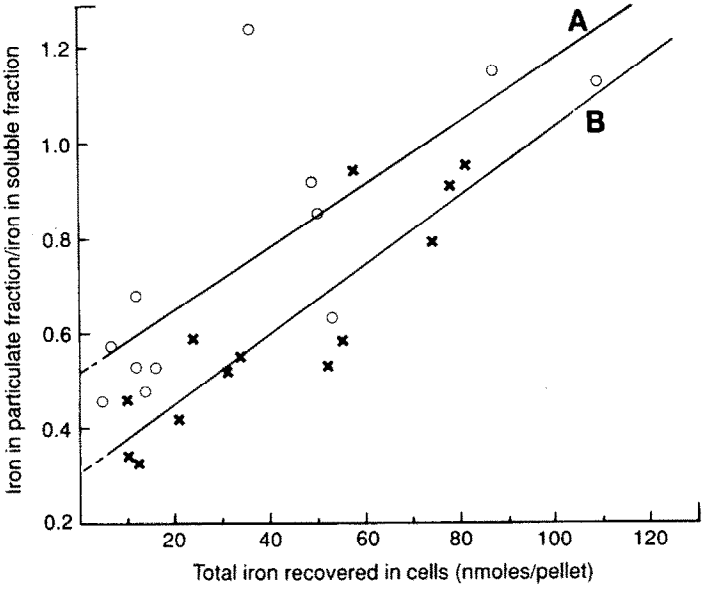

Fig. 3. Comparison of iron distribution in the particulate and soluble fractions of *B. thermophilum* under normal conditions (curve B, ×——×) and in the presence of 200 μM Mg^{2+} (curve A, ○——○). Cells were incubated in the presence of various iron concentrations at 37°C for 60 min.

into three likely phases, viz. surface binding, internalization (via a putative permease) and binding in the cell interior, we can then inquire as to the site of action of Mg^{2+}. Experiments at 0°C and with the cell particulate fraction (Tables 1 and 2) have shown that Mg^{2+} does not affect the initial cell surface binding of Fe^{2+}. Nor does Mg^{2+} affect the binding of iron to the normally masked sites that can be opened up by protoplasting the cells. Mg^{2+} does not affect in any major way the binding of iron in the cell interior, as is indicated in Fig. 3 and confirmed by displacement experiments shown in Table 3.

The most likely effect of Mg^{2+} is then at the level of the iron transport mechanism (permease). This

mechanism is directly or indirectly associated with the action of a transport ATPase (Bezkorovainy *et al.*, 1986). Mg^{2+} is associated with the function of such ATPases (Pedersen, 1982), and it is then understandable why lactate production goes up when [Mg^{2+}] increases: increased ATPase activity increases the demand and utilization of primary fuels such as glucose. Why this results in an inhibition of iron uptake remains a mystery, unless the iron permease, associated with the ATPase, is also an Mg^{2+} transporter. It would indeed be surprising if both Mg^{2+} and Fe^{2+} but not Ca^{2+} were transported into the cell by the same permease, since the atomic size of Fe^{2+} is much closer to that of Ca^{2+} than that of Mg^{2+}. As shown in Table 4, Ca^{2+} had a mild stimulatory effect on iron uptake by the cells, most likely via its effects on cell plasma membrane fluidity, and had little if any effect on the inhibitory action of Mg^{2+}.

Figure 3 shows that the effects of Mg^{2+} on iron uptake by the cells were reversible, and such reversal could be easily affected by removing Mg^{2+} from the cellular suspension medium. The kinetics of iron uptake were, unfortunately, too complex to permit the construction of Lineweaver–Burk type plots, from which the type of inhibition by Mg^{2+} could be determined unambiguously. However, given the ease with which iron uptake resumes following Mg^{2+} removal from the medium, it would not be unreasonable to suggest that it may be of competitive nature. It would also be attractive to speculate that Mg^{2+} may be a physiological modulator of iron uptake by bifidobacteria.

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