Uptake of Iron by \textit{Bifidobacterium thermophilum} Depends on the Metal Content of Its Growth Medium

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\textbf{ABSTRACT}

\textit{Bifidobacterium thermophilum} was grown in the trypticase-protease-yeast extract medium containing different concentrations of Mg$^{2+}$, Ca$^{2+}$, and Fe$^{3+}$, and Fe$^{2+}$ uptake was then measured in cells grown in the presence of metal ions. Cells that grew in metal-depleted media took up more iron than was the case with cells grown in metal-replete media. Such differences were not due to a necessity to transport iron against a concentration gradient, because soluble iron content was the same in cells grown in all types of media. Moreover, iron uptake could be diminished in cells grown in the presence of increased amounts of Ca$^{2+}$ or of Mg$^{2+}$. Iron uptake in cells grown in metal-depleted media was dependent on iron concentration, and the uptake was virtually abolished at 0°C and after heating the cells at 65°C for 20 min. A possible explanation for this behavior is that the metals suppress the expression of a metal transporter.

(Key words: \textit{Bifidobacterium thermophilum}, iron, ferrous, metals)

Abbreviation key: $A_{410}$ = absorbance at 610 nm wavelength, TPY = trypticase-phytone-yeast extract.

\textbf{INTRODUCTION}

There are over 20 species in the genus \textit{Bifidobacterium}. Many have been isolated from human sources, whereas others seem to prefer animal habitats [see reviews by Bezkorovainy and Miller-Catchpole (1) and by Rasic and Kurmann (9)]. \textit{Bifidobacterium thermophilum} is a species that has been isolated from cattle rumen (6, 13). In contrast to many other bifidobacterial species, \textit{B. thermophilum} grows well at relatively high temperatures (e.g., 43°C), it does not ferment lactose, and it forms protoplasts with relative ease (5).

Iron uptake and other physiologic studies have been performed on \textit{Bifidobacterium breve} and \textit{Bifidobacterium bifidum} in our laboratory, and a hypothesis on their function in human physiology was formulated. It was proposed that intestinal bifidobacteria in infants and possibly in adults act in a host-defense capacity by withholding iron from pathogenic microorganisms (3, 4). No substantial iron metabolism or other physiologic studies have been performed on bifidobacteria of animal origin, such as \textit{B. thermophilum}, and no specific function has been assigned to these organisms in animal physiology. Rumen bacterial physiology has been the subject of recent reviews [e.g., (11)].

The purpose of the current work was to initiate physiologic and nutritional studies on \textit{B. thermophilum} as a convenient representative species of bifidobacteria from domestic animals. The ease with which it forms protoplasts makes this species especially attractive for such studies. In this work, we report that the accumulation of iron by \textit{B. thermophilum} depends on the metal content of the medium in which the cells were grown.

\textbf{MATERIALS AND METHODS}

Microorganisms and Their Media

Lyophilized \textit{B. thermophilum} (ATCC 25866) was purchased from American Type Culture Collection (Rockville, MD). Cultures were routinely monitored for fructose-6-phosphate phosphoketolase activity according to Scardovi (12). This enzyme is unique to bifidobacteria.

\textit{Bifidobacterium thermophilum} was grown in the trypticase-phytone-yeast extract (TPY)
medium prepared according to Scardovi (12). To obtain a modified TPY medium with lesser transition metal concentrations, certain alterations were made in the TPY medium as follows: K2HPO4, MgCl2·H2O, ZnSO4·7H2O, CaCl2, and FeCl3 were omitted, and the following, not present in the TPY medium, were added (per liter): 1 g of imidazole, 10 g of sodium acetate, and .5 g of KCl. To decrease metal concentration even further, the modified TPY medium was treated by Chelex 100 (Bio­rad Laboratories, Richmond, CA), an insoluble heavy metal chelator. Twenty grams of Chelex 100 were added per 500 ml of modified TPY medium, the slurry was stirred for 30 min, and the Chelex 100 removed by filtration. The filtrate was termed chelated TPY. All media were adjusted to pH 6.5 and autoclaved as described by Scardovi (12).

Iron Uptake Experiments

For the purpose of performing iron uptake experiments, cell cultures were chilled on ice and centrifuged at 4°C for 10 min. The pellets were washed once with ice-cold modified Hanks solution consisting of .4 g of KCl, 8 g of NaCl, .14 g of CaCl2, 8.2 g of sodium acetate, and 2 g of glucose/L, adjusted to pH 5.0. In previous work (3, 4), lactose was used instead of glucose. However, B. thermophilum does not ferment lactose. After washing, cells were suspended in the modified Hanks solution to give a final absorbance at 610 nm wavelength (A610) of .7 to .9. Iron uptake experiments were done using radioactive ferrous iron in concentrations of 6 to 400 μM according to procedures previously published (2, 4). Washing of cells after incubation with iron was done at 0°C with .2 M acetate buffer at pH 5.0 followed by .1 M HEPES-5 mM EDTA at pH 7.0. Further washing failed to remove significant quantities of iron from the cell pellets. Iron content of cell pellets was then determined by counting in the Beckman Gamma-4000 counter (Beckman Instruments, Palo Alto, CA).

Analytical Procedures

Lactate was occasionally determined after iron uptake. This was done with lactate dehydrogenase-NAD kits available from Sigma (St. Louis, MO). Deoxyribonucleic acid was determined spectrophotometrically at 260 nm according to Roby and White (10). Iron was determined colorimetrically with ferrozine using kits available from Sigma Corporation (St. Louis, MO). Calcium and Mg were determined in the Actachem solid-phase instrument manufactured by Eastman Kodak Co. (Rochester, NY). Metals were determined in the soluble fractions of bacteria after sonications and removal of the cell wall and membrane fraction by centrifugation. Results were expressed as a ratio of milligrams or milliequivalents of metal per mole DNA.

Materials

All common chemicals were purchased from Fisher Laboratories (Chicago, IL). Labeled ferrous iron was the product of ICN Corp. (Irvine, CA). Specialty chemicals such as vitamins and enzymes were purchased from Sigma (St. Louis, MO), whereas peptone and trypticase were purchased from BBL Microbiology Systems (Cockeysville, MD).

RESULTS

Iron Uptake by Microorganisms as a Function of Time

The microorganism was grown under anaerobic conditions in batches of 120 ml for 24 h as previously described (2, 3, 4, 8). Cell densities (A610) were typically 1.4, 1.2, and .90 in the TPY, modified TPY, and chelated TPY media, respectively.

Microorganisms grown in the three media were suspended in the modified Hanks solution with A610 being adjusted to comparable levels (.70 to .90), and iron uptake experiments were performed. No significant iron uptake was observed if glucose in the modified Hanks solution was substituted by lactose (data not shown). Typical results are shown in Figure 1. Organisms grown in iron-replete media were poor iron transporters, whereas those grown in iron-depleted media accumulated large amounts of iron. In addition, iron uptake was biphasic: a rapid accumulation for the first 20 min followed by a slower uptake. Considerable amounts of iron were also bound to the cell within 1 min of starting the experiment. In case of curve E, this amounted to about 20 nmol per pellet, i.e., about 10% of iron ac-
Iron accumulation in *Bifidobacterium thermophilum* as a function of high iron concentration in the medium after 60 min of incubation with radioactive ferrous iron at 37°C. Curve A refers to bacteria grown in the trypticase-phytone-yeast extract (TPY) medium; curve B, in modified TPY medium; and curve C, in chelated TPY medium. In all cases, absorbance at 610 nm was .700.

![Graph](image)

**Figure 2.** Iron accumulation in *Bifidobacterium thermophilum* as a function of high iron concentration in the medium after 60 min of incubation with radioactive ferrous iron at 37°C. Curve A refers to bacteria grown in the trypticase-phytone-yeast extract (TPY) medium; curve B, in modified TPY medium; and curve C, in chelated TPY medium. In all cases, absorbance at 610 nm was .700.

Cumulated in 2 h. No evidence of a plateau was observed after 2 h in cells grown in the modified and chelated TPY media.

**Iron Uptake by Microorganisms as a Function of Iron Concentration**

Iron uptake by *B. thermophilum* was dependent on iron concentration in the medium. Figure 2 depicts these relationships at relatively high iron concentrations. Low iron concentrations were those in the 5 to 40 μM range. High concentrations were defined as those between 50 and 400 μM (3, 4).

The results of these studies are expressed simply as the amount of iron accumulated in 60 min as a function of iron concentration in the medium. In our previous studies with *B. breve* and *B. bifidum* (2, 3, 4), the results depicted in Figures 2 and 3 could be represented by double reciprocal plots using iron accumulations or initial uptake velocities. Such plots were linear, and apparent Michaelis constants could be calculated. In the case of *B. thermophilum*, reciprocal plots were also linear; however, such curves tended to intersect the x-axis on its positive side and the y-axis on its negative side (data not shown). Interpretation of such curves would have been ambiguous at best.

Figures 2 and 3 do, nevertheless, show that iron accumulation is dependent on outside iron concentration and that accumulation depends on metal content of the medium in which the cells were grown.

**Iron Uptake by Organisms at 0°C and After Heating**

Figure 4 indicates iron accumulation by *B. thermophilum* at 0°C. It is clear that, for organisms grown in the modified and chelated TPY media, the uptake was considerably lower than that at 37°C (see Figure 2). Iron accumulation...
IRON UPTAKE BY BIFIDOBACTERIUM

Figure 3. Iron accumulation in *Bifidobacterium thermophilum* as a function of low iron concentration in the medium after 60 min of incubation with radioactive ferrous iron at 37°C. Curve A refers to bacteria grown in the tryptcase-phytone-yeast extract (TPY) medium; curve B, in modified TPY medium; and curve C, in chelated TPY medium. In all three cases absorbance at 610 nm was .900.

at 0°C by cells grown in the TPY medium differed but little from that at 37°C (Figure 2).

Bacterial cells grown in the modified TPY medium were heated at 65°C for 20 min. As indicated by Figure 5, iron uptake by such cells became less than 10 nmol per pellet. Lactate determination, after 60-min incubation time, showed a concentration of 38.8 µg/ml in the medium of the heated cells and 125.8 µg/ml in that of normal cells. Heated cells thus had lost most of their glucose-metabolizing and iron-accumulating capacities.

Metal Content of Cells and Their Growth Media

Table 1 indicates metal content in the three growth media before and after cell growth. It is seen that Fe, Ca, and Mg contents of the modified TPY medium are some 10-fold lower than those of the TPY medium and lower yet in the chelated TPY medium. Spent media Fe and Ca concentrations are comparable with those of respective fresh media, although Mg concentrations in the spent media were lower than those of fresh media.

Figure 4. Iron accumulation in *Bifidobacterium thermophilum* as a function of iron concentration in the medium after 60 min of incubation at 0°C. Curve A refers to bacteria grown in the tryptcase-phytone-yeast extract (TPY) medium; curve B, in modified TPY medium; and curve C, in chelated TPY medium. Absorbance at 610 nm for curve A was .900 and .700 for curves B and C.

Figure 5. Iron accumulation in *Bifidobacterium thermophilum* grown in the modified tryptcase-phytone-yeast extract medium after heating at 65°C for 20 min as a function of iron concentration in the medium (curve A). Curve B represents the results in the unheated cells. Accumulation shown is after 60 minutes at 37°C. Absorbance at 610 nm was .900.
TABLE 1. Metal composition of fresh and spent bacterial growth media and of soluble fractions of Bifidobacterium thermophilum.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Fresh TPY</th>
<th>Spent TPY</th>
<th>Fresh Modified TPY</th>
<th>Spent Modified TPY</th>
<th>Fresh Chelexed TPY</th>
<th>Spent Chelexed TPY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Spent</td>
<td>Fresh</td>
<td>Spent</td>
<td>Fresh</td>
<td>Spent</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Fe</td>
<td>95.9 ± 1.9</td>
<td>95.3 ± 1.9</td>
<td>9.50 ± 1.4</td>
<td>11.9 ± 0.7</td>
<td>6.60 ± 0.5</td>
<td>4.50 ± 1.7</td>
</tr>
<tr>
<td>Ca</td>
<td>4.83 ± .06</td>
<td>4.43 ± .25</td>
<td>.450 ± .07</td>
<td>.440 ± .05</td>
<td>&lt;.10</td>
<td>&lt;.10</td>
</tr>
<tr>
<td>Mg</td>
<td>8.27 ± .23</td>
<td>7.20 ± .52</td>
<td>1.05 ± .07</td>
<td>&lt;.04</td>
<td>.240 ± 0</td>
<td>&lt;.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe, µM mol/nmol DNA</td>
<td>2.542 ± .08</td>
<td>3.040 ± .923</td>
<td>3.909 ± .173</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca, mg/mol DNA</td>
<td>&lt;.10</td>
<td>&lt;.10</td>
<td>&lt;.10</td>
<td>&lt;.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg, meq/mol DNA</td>
<td>398.7 ± 95.3</td>
<td>65.11 ± 10.6</td>
<td>239.4 ± 54.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1TPY = Trypticase-phytoe-yeast extract.
2Quantitation limit was .1 mg/dl.
3Quantitation limit was .04 meq/L.
4Cells grown in the media indicated.

Calcium content of bacterial cell extract was too low to be quantitated. Iron content tended to be somewhat higher in cells grown in iron-depleted media; however, the difference between cells grown in the TPY and modified TPY media was not statistically significant. The difference between iron content of cells grown in the TPY and chelated modified TPY media was significant. Magnesium content of cell extracts was unusual; although those of cells grown in the TPY and chelated modified TPY media differed but slightly from each other, that of cells grown in the modified TPY medium was 5- to 7-fold lower.

Iron Uptake by EDTA- and Pronase-Treated Cells

To determine whether iron uptake by the cells may be dependent on surface-bound iron originating from the bacterial growth medium, the cells were washed, after harvesting, first with .1 M HEPES-5 mM EDTA at pH 7.0 and then with the modified Hanks solution at 0°C. Iron uptake experiments were then performed at 37°C. Using cells grown in the modified TPY medium, no significant differences were observed between the EDTA washed and regular cells following incubations with 100 to 400 µM Fe²⁺ for up to 60 min (data not shown). Cells grown in the modified TPY medium were also subjected to pronase (nonspecific protease) digestion at 37°C for 30 min. Pronase concentration was 5 mg/ml, and the incubation medium was .1 M phosphate buffer at pH 7.0 containing 2 mg/ml glucose. Following this, iron uptake studies were done at pH 5 using iron concentrations of 200 to 400 µM. There was no decline in iron uptake after 60 min in the pronase-treated cells (data not shown). Preliminary data have indicated that iron uptake may, in fact, increase following the pronase treatment.

Iron Uptake by Cells Grown In Modified TPY Media Reconstituted with Metals

Modified TPY medium contains no exogenously added metal salts, but the TPY medium contains added quantities of Fe²⁺, Ca²⁺, Zn²⁺, and Mg²⁺ (12). It would, therefore, be of interest to determine which metal or metals are responsible for low iron uptake by cells grown in the TPY medium. Consequently, individual metal salts were added to the modified TPY medium in quantities shown in Table 2, and cells grown in such media were then tested for iron uptake.

No cell growth occurred in the presence of ZnSO₄ alone. This is expected, because Zn²⁺...
TABLE 2. Uptake of Fe\(^{2+}\) by cells grown in the presence of various metals in modified trypticase-phytone-yeast extract medium and the effect on Fe\(^{2+}\) uptake by the presence of Ca\(^{2+}\) and Mg\(^{2+}\) (in percentage of control).

<table>
<thead>
<tr>
<th>Metal compound added to growth medium</th>
<th>Concentration</th>
<th>Growth(^a)</th>
<th>Fe(^{2+}) Uptake</th>
<th>Compound present in Fe(^{2+}) uptake experiments(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\bar{X})</td>
<td>SD</td>
<td>Concentration (\bar{X}) SD</td>
</tr>
<tr>
<td>FeCl(_3)</td>
<td>450 µg/dL</td>
<td>.98</td>
<td>.13</td>
<td>Fe(^{2+})</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>.015 g/dL</td>
<td>.94</td>
<td>.11</td>
<td>None</td>
</tr>
<tr>
<td>MgCl(_2)-6H(_2)O</td>
<td>.05 g/dL</td>
<td>1.01</td>
<td>.09</td>
<td>None</td>
</tr>
<tr>
<td>CaCl(_2) and MgCl(_2)-6H(_2)O</td>
<td>.015 and .05 g/dL</td>
<td>.94</td>
<td>.09</td>
<td>None</td>
</tr>
<tr>
<td>None added(^c)</td>
<td>...</td>
<td>1.06</td>
<td>.15</td>
<td>CaCl(_2)</td>
</tr>
<tr>
<td>None added(^d)</td>
<td>...</td>
<td>Same as control</td>
<td>101</td>
<td>CaCl(_2)</td>
</tr>
<tr>
<td>None added(^e)</td>
<td>...</td>
<td>Same as control</td>
<td>&lt;1</td>
<td>MgCl(_2)-6H(_2)O</td>
</tr>
<tr>
<td>None added(^f)</td>
<td>...</td>
<td>Same as control</td>
<td>56</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^1\)Iron uptake from 300 µM Fe\(^{2+}\) for 60 min at 37°C. Standard deviations, where indicated, calculated on the basis of at least four experiments.

\(^2\)Absorbance at wavelength of 610 nm.

\(^3\)These were in addition to components present in the modified Hank's solution, as described in Materials and Methods.

\(^4\)This served as a reference point (control).

\(^5\)Iron uptake done in modified Hank's solution from which Ca\(^{2+}\) was omitted.

is a bifidobacterial growth inhibitor whose inhibitory action (as is the case in with TPY medium) is negated by iron (14). In all other cases, the cells grew as expected, and the iron uptake results are summarized in Table 2. This table shows that growing cells in the presence of added Ca\(^{2+}\), Fe\(^{3+}\), and Mg\(^{2+}\) resulted in drastic declines in their ability to take up Fe\(^{2+}\). The effect of Ca\(^{2+}\) was least pronounced. Within each metal category, these effects were dose-dependent.

Table 2 also indicates the uptake of iron in the presence of Mg\(^{2+}\) and Ca\(^{2+}\) by cells grown in the modified TPY medium, i.e., without adding any metal salts. The Mg\(^{2+}\) had an inhibitory effect on iron uptake, as previously observed with B. breve (3). Addition of Ca\(^{2+}\) to the iron uptake assay medium (modified Hank's solution) remained without effect; however, when Ca\(^{2+}\) was omitted altogether from the modified Hank's solution, iron uptake was cut almost in half.

DISCUSSION

Physiological properties of B. thermophilum grown in metal-depleted media and identified herein may be compared with those of B. breve and B. bifidum determined on previous occasions (2, 3, 4). In all cases, iron uptake was dependent on iron concentration in the medium, and iron uptakes were substantial, i.e., 100 to 300 µmol per pellet at iron concentrations of 300 µM. Moreover, in all cases, the cellular metabolic machinery was apparently required, as indicated in case of B. thermophilum, by lack of iron uptake at 0°C and by heated cells, and by cells deprived of glucose. There were some differences as well. For example, the mode of iron uptake by B. breve and B. bifidum was relatively simple and could be represented by double reciprocal plots, but in B. thermophilum this appeared to be more complex.

Time course studies on iron uptake by B. thermophilum have shown that considerable amounts of iron were bound to the cells almost instantaneously, most likely representing a surface phenomenon. This initial binding of iron was followed by a biphasic accumulation, which continued beyond the 2-h time limit shown in Figure 1.

All three phases of iron accumulation were greatly diminished when the cells that were used for iron uptake experiments were grown in the regular TPY medium, which contains
added Fe^{3+}, Ca^{2+}, Zn^{2+}, and Mg^{2+}. Conversely, iron uptake was greatest in cells grown in media with the lowest metal concentration. The difference was even observed at 0°C, where iron transport into cell interior should not take place. This is indicative of differences in cell surface iron binding. It was, however, impossible to alter such putative iron-binding structures by pronase digestion.

There are two possibilities for why iron uptake increases in cells grown in media with progressively lower metal concentrations: 1) cellular iron transport mechanisms may be induced by low iron concentrations in the medium and 2) cells grown in iron-replete media may become so iron-loaded that they are simply unable to transport additional iron against such a high iron concentration gradient. The latter possibility was tested by measuring iron concentrations in fresh and spent growth media as well as the soluble portion of the cells (Table 1). Such data indicated that there was no substantial iron removal from the media during growth and that iron concentrations in the soluble portions of the cells, if anything, increased as growth media became iron poor. Treatment of cells grown in iron-replete media with EDTA prior to iron uptake experiments had no effect on iron behavior. We can thus discount possibility 2 above and must consider possibility 1 as worthy of further consideration.

This proposal is strengthened by the fact that iron uptake could be suppressed in cells grown in media with increased Ca^{2+} or Mg^{2+} levels (Table 2). Apparently, certain metals, when present in sufficiently large amounts in the growth medium, are capable of suppressing the iron uptake, possibly by suppressing the expression of an iron transport particle. It is curious, however, that Ca^{2+} would suppress the expression of such a putative transporter but would not interfere with iron transport per se. In fact, our data suggest that Ca^{2+} may be required for optimal iron transport; however, Mg^{2+} interferes with iron transport as expected. Further work using recombinant DNA techniques would be necessary to test our hypothesis.

It is impossible to state whether or not the presence of metals other than Fe^{3+}, Ca^{2+}, and Mg^{2+} during growth would later suppress the uptake of iron by *B. thermophilum*. Most transition metals inhibit bacterial growth at higher than trace concentrations. Finally, it may be recalled that the expression of siderophores in other microorganisms is also regulated by iron content of their growth media (7). Bifidobacteria, however, produce no siderophores, so far as is known.

**REFERENCES**