Distribution of Accumulated Iron in *Bifidobacterium thermophilum*

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Bifidobacterium thermophilum (ATCC 25866) is capable of absorbing large amounts of Fe^{2+} from a buffer solution at pH 5 containing glucose if the organism had been grown in an iron-poor medium. An investigation of the distribution of such iron in cell was undertaken, where the cells were disrupted by sonication for 4 min. Iron was thus measured on the cell surface, in its particulate fraction and its soluble fraction. Iron distribution depended on total cellular iron content: at low iron loads, the Fe^{2+} was present largely on the cell surface and in the soluble fraction. At higher Fe^{2+} levels, the particulate fraction acquired progressively larger supplies of iron. Iron was bound by both the surface and cytosol sides of the plasma membrane in the intact cell; however, the latter ability was lost following cell disruption. Surface binding of iron both in intact cells at 0 °C and in particulate fraction at or 37 °C was biphasic: there were rapid iron binding and slow iron binding sites. The latter was heat sensitive. Iron from the former could be eluted by 2 mM cold Fe^{2+}. On the basis of Scatchard plots, the particulate fraction could bind a maximum of about 30 nmol of iron/mg of protein with a dissociation constant of about 735 mM at 37 °C at the rapid binding site.

INTRODUCTION

Bifidobacteria have long been advocated as a means of supplementing various milk products (Kroger et al., 1989). These organisms may originate from human or animal sources, but all share the same general properties: They are Gram-positive rods, catalase-negative, and non-spore-forming, and they ferment glucose to acetic and lactic acids. The latter property is believed to be beneficial to the host, since the organic acids produced serve to inhibit the proliferation of pathogens in the intestinal tract (Bezkorovainy and Miller-Catchpole 1989).

Bifidobacterium thermophilum (*B. thermophilum*) is a normal cattle rumen microorganism (Mitsuoka, 1969). Our previous work has shown that these organisms take up remarkably large quantities of iron at 37 °C and a much smaller quantity at 0 °C. Moreover, it was shown that the extent to which iron is accumulated by *B. thermophilum* depends on the iron content of the medium in which it was grown: When iron concentrations therein were relatively high as mandatd by the TPY medium (Sardovi, 1986), there was little, if any, iron uptake. On the other hand, iron uptake was quite substantial when iron in the growth medium was restricted to 10 μM or less (Kot and Bezkorovainy, 1991). Further information on this and other bifidobacterial species can be obtained from a recent monograph by Bezkorovainy and Miller-Catchpole (1989).

Bifidobacteria accumulate iron when it is presented in the ferrous oxidation state. This form of iron would be expected under anaerobic conditions such as are prevalent in the human colon or cattle rumen. We suggested that iron accumulation by bifidobacteria under these conditions may serve a host defense function, preventing the growth of pathogens for want of iron (Bezkorovainy and Solberg, 1989). One question has always been the distribution of the absorbed iron in the cell. The work described in this paper has addressed this issue by determining the location of iron on the surface and in the soluble and particulate fractions of the cell.

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MATERIALS AND METHODS

Microorganisms. *B. thermophilum* (ATCC 25866) was obtained from American Type Culture Collection (Rockville, MD). The organism may be propagated and grown in the TPY medium (Scardovi, 1986) or in the iron-depleted TPY medium according to Kot and Bezkorovainy (1991). The latter was termed "modified TPY medium". Unless otherwise stated, all organisms were grown in the latter medium. For quality-control purposes, the organisms were routinely examined microscopically using the Gram stain and tested for the presence of phosphofructoketolase, which is unique to bifidobacteria (Scardovi, 1986). Occasionally, the identity of the organisms was confirmed by sugar fermentation studies performed in a local clincal microbiology laboratory. The organism was propagated for at least a year without observation of any alterations in the above criteria or iron uptake patterns.

The number of organisms present in the growth medium was estimated by diluting cultures serially and then inoculating blood agar plates (Remel anaerobic blood agar, CDC formulation, Lenexa, KS) with calibrated loops. Growth was permitted to take place under anaerobic conditions for 72 h, and then the colonies were counted on a counting grid. A culture with an A_{610} = 1.2 contained 8.5×10^7 to 1×10^8 organisms/mL. A "pellet", a term used to express iron uptake results, is defined as the organisms from 5 mL of a bacterial culture or suspension with an A_{610} = 1.2. It thus represents between 4×10^8 and 5×10^8 organisms.

Analytical Procedures. Protein determinations were done according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Phosphorus was determined according to a modification of the Fiske-SubbaRow method (Lowry et al., 1954). The accuracy of this determination was checked by phosphorus determination in κ-casein, which contains 0.14–0.35% phosphorus (Waugh, 1971). Lactic acid was determined with lactate dehydrogenase using kits supplied by Sigma Chemical Co. (St. Louis, MO). Radioactivity was measured in a Beckman-Gamma 4000 counter (Beckman Instruments, Palo Alto, CA).

Iron Uptake Determinations. Iron was taken up by cells in the air at either 0 or 37 °C from the "modified Hanks" solution at pH 5 using $^{59}Fe^{2+}$. Alternatively uptake was carried out in 0.1 M 3,3-dimethylglutarate buffer, which was effective at both pH 5.0 and 6.5. Other ingredients were identical to those of the modified Hanks solution. The modified Hanks solution was described in detail by Kot and Bezkorovainy (1991). Washing of cells was done with 0.2 M acetate buffer at pH 5 and 0 °C. Whenever it was desired to remove the $^{59}Fe^{2+}$ from the cell surface

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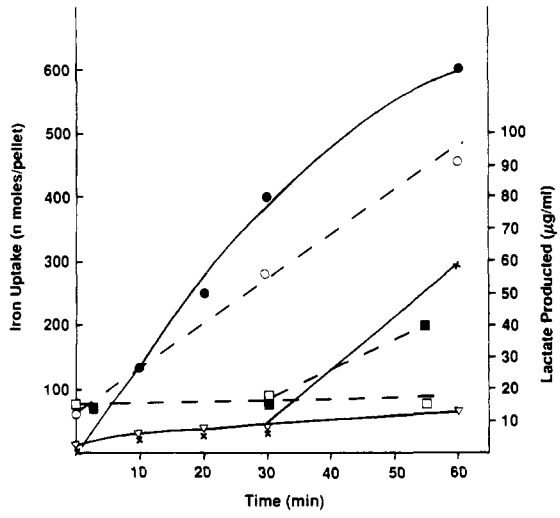

Figure 1. Iron uptake and lactate production by *B. thermophilum* in the presence and absence of glucose (2 mg/mL) at 37 °C (●) Normal iron uptake in the presence of glucose; (▽) iron uptake in the absence of glucose; (×) iron uptake in the absence of glucose, where glucose was added after 30 min; (○) normal lactate production in the presence of glucose; (□) lactate production in the absence of glucose; (■) lactate production in the absence of glucose, where glucose was added after 30 min. A pellet represents cells from a 5-mL suspension with an $A_{610} = 1.2$. Iron concentrations used were 270–285 μM. The cells were initially grown in the modified TPY medium.

or from the particulate fractions, the washing was performed in the presence of nonradioactive (cold) 2 mM ferrous sulfate at 0 °C for 30 min.

Sonication. Disruption of cells could be accomplished by sonication with a Virtis Co. (Gardiner, NY) Virsonic Model 16-850 cell disrupter at 60% of maximum using the intermediate probe. Cell suspensions in a plastic tube were kept ice-cold, and sonication was continued for 2–8 min. Cells for sonication purposes were suspended in modified Hanks at pH 5. This buffer was chosen to avoid the oxidation of Fe^{2+} to Fe^{3+} present in the cells. It is well-known that such oxidation is faster at pH 7 than at pH 5. However, for the measurements of phosphofructoketolase activity, sonication was done on cells suspended in 0.1 M phosphate buffer at pH 7. Phosphofrutoketolase was found to lose activity irreversibly at pH 5. Following sonication, the preparation was centrifuged at 28000*g* for 30 min. The sediment was termed the particulate fraction and the supernatant, the soluble fraction. The former consists largely, if not entirely, of cell wall and plasma membrane fragments.

Materials. Unless otherwise noted, all chemicals used herein were purchased from Sigma. Exceptions were peptone and trypticase, which were obtained from BBL Microbiology Systems (Cockeysville, MD). $^{59}Fe^{2+}$ was obtained from Du Pont Laboratories (Boston, MA).

RESULTS

Normal Iron Uptake and Lactate Production by *B. thermophilum*. *B. thermophilum* suspensions took up $^{59}Fe^{2+}$ in an energy-dependent manner as shown in a typical case in Figure 1. If glucose was excluded, little iron was taken up and little lactate was produced. As glucose was added to an energy-starved cell suspension, iron uptake and lactate production were resumed.

Binding of $^{59}Fe^{2+}$ by *B. thermophilum* at 0 °C. Cells grown in both the TPY and modified TPY media were subjected to iron binding studies at 0 °C as a function of iron concentration, and the results are shown in Table I. Iron binding was biphasic in all cases, and regression values (*y*-intercepts) were deemed to represent a rapid binding of $^{59}Fe^{2+}$. The amount of $^{59}Fe^{2+}$ that could be washed off with 2 mM cold Fe^{2+} was remarkably similar to the regression values and depended on the amount of iron that was present in the cell suspension medium. However, it was independent of time in the 10–60-min range used in this work (data not shown). It is assumed that no $^{59}Fe^{2+}$ was internalized by the cells at 0 °C and that all binding of $^{59}Fe^{2+}$ represented a surface phenomenon.

Sonication and Composition of the Particulate Fraction. To ascertain the optimum sonication time, cells were disrupted by 2-, 4-, 6-, and 8-min sonication. Lactate production as a function of time was determined, as were protein and phosphorus values of the particulate fraction. Lactate production would be indicative of the presence of intact organisms. The results are indicated in Table II. It shows that the 2-min sonicate produced lactic acid, which increased with time, whereas sonication for ≥4 min resulted in little or no lactate production. Total protein and phosphorus recovered in the particulate fraction declined with time. Thus, increased sonication times not only disrupted the cells (as indicatd by the lack of lactic acid production) but also dispersed the particulate fraction. An optimum sonication time would thus leave no intact cells in place, yet permit maximum integrity of the particulate fracation. Such an optimum sonication time appeared to be 4 min. Sonication was thus performed in a 0 °C bath in 2-min bursts interspersed with a 2-min rest period.

Protein and phosphorus contents of the particulate fraction were measured on a number of preparations, and there was considerable batch-to-batch variation with regard to both protein and phosphorus contents. For 10 samples, the average particulate fraction protein content was 1.55 ± 0.373 mg and phosphorus content was 0.434 ± 0.00835 mg/pellet, while the protein to phosphorus ratio was 32.3 ± 7.15. Because of such variation, iron binding by the particulate fraction is expressed on the basis of milligrams of protein rather than pellet.

Distribution of Iron in *B. thermophilum*. The distribution of iron following loading with $^{59}Fe^{2+}$ was investigated in three cellular compartments: surface, particulate fraction, and soluble fraction. Surface binding was determined by first washing the iron-loaded cells at 0 °C with 0.2 M acetate buffer at pH 5.0 and then with the same buffer containing 2 mM cold Fe^{2+} for 30 min. It thus represented only the exchangeable portion of surface-bound iron (see Table I). Following this, the cells were washed with the 0.2 M acetate buffer without the iron and then sonicated. The results, at two pH values are depicted in Tables III and IV.

First, it will be noted that there is more lactate produced and iron taken up by whole cells at pH 6.5 than at 5.0, with the exception that at low outside iron concentrations iron uptake at pH 5.0 was higher than that at pH 6.5. The difference was not particularly significant, however. The most important finding was that at both pH values the percentage of particulate fraction-bound iron increased with increasing outside iron concentration, while that of the soluble fraction decreased (Table III). This was especially visible at pH 5.0, while at pH 6.5 the percent of iron in the cytosol fraction remained fairly constant at 60–70%. The absolute amount of iron in the two compartments was, of course, increased as iron load increased (Table III). Another trend, observed at both pH values, was that the proportion of iron elutable from the particulate fraction with 2 mM cold Fe^{2+} declined as particulate fraction-bound iron increased. Thus, at lowest outide iron concentrations, 55–67% of particulate fraction-bound iron could be eluted, while at higher concentrations this was only about 25% (Table III).

Table I. Iron Binding by *B. thermophilum* Cells at 0 °C (in Nanomoles/Pellet)

medium in which cells were grown	[$^{59}Fe^{2+}$], μM	time, min				straight line correlation coefficient[a]	linear regression to 0 time, nmol/pellet	washed off by 2 mM cold Fe^{2+},[b] nmol/pellet
		10	20	30	60			
TPY	53.0	0.750	0.970	0.990	1.59	0.99	0.580	0.640
	106	1.63	2.65	1.92	3.19	0.80	1.56	1.14
	210	2.99	3.31	3.42	5.74	0.97	2.17	2.27
	316	4.34	4.64	5.67	7.28	0.99	3.66	2.50
modified TPY	56.0	3.41	4.27	4.84	7.25	0.99	2.66	3.20
	114	7.05	8.81	9.09	14.3	0.99	5.53	5.77
	206	12.1	15.2	18.0	24.8	0.99	10.0	10.5
	302	20.0	25.0	26.0	37.4	0.99	17.0	15.9

[a] Binding (*y*-axis) vs time (*x*-axis). [b] 60-min sample.

Table II. Properties of *B. thermophilum* Particulate Fraction as a Function of Sonication Time

time of sonication, min	lactate production,[a] mg/mL	iron uptake[b]		washed off with cold 2 mM Fe^{2+}, %	protein content, mg/original pellet	phosphorus, mg/original pellet	protein/phosphorus ratio
		nmol/original pellet	nmol/mg of protein				
0[c]	77.8	237	72.9	14.8	3.25	0.130	25.0
2	15.0	105	49.3	13.4	2.13	0.0700	30.4
4	3.1	43.6	25.2	14.7	1.73	0.0450	38.4
6	0	22.7	15.8	21.1	1.44	0.0400	36.0
8	0	28.2	16.8	20.6	1.68	0.0425	39.5

[a] 60-min incubation; all values obtained were corrected for those given by 185 μM Fe^{2+} solutions (controls). [b] Outside iron concentration was 185 ± 4.41 μM; 60-min incubation modified Hanks. [c] Whole cells; protein and phosphorus values are a sum of both soluble and particulate fractions.

Table III. Distribution of Iron in *B. thermophilum* following Loading with $^{59}Fe^{2+}$ (Incubation Times, 60 min at 37 °C; Cells Were Disrupted by Sonication for 4 min)

[^{59}Fe] in loading process, μM	total uptake, nmol/pellet	recovery[a]	buffer and pH	washed off by 2 mM cold Fe^{2+} [b]	particulate fraction	washed off by 2 mM cold Fe^{2+} [c]	soluble fraction	lactate produced,[d] μg/mL
25	13.1	10.2[e] (78)[f]	Ac,[g] pH 5	1.69[e] (16.6)[f]	2.68[e] (26.3)[i]	1.80[e] (67.2)[j]	5.87[e] (57.5)[i]	
50	40.7	33.7 (83)	Ac,[g] pH 5	5.11 (15.2)	102.2 (30.3)	5.73 (56.2)	18.4 (54.6)	
97	105	77.8 (74)	Ac,[g] pH 5	8.50 (10.9)	32.9 (42.3)	11.9 (36.2)	36.4 (46.8)	
196	253	203 (80)	Ac,[g] pH 5	13.6 (6.70)	118 (58.1)	30.2 (25.6)	71.5 (35.2)	
29	13.3	12.4 (91)	Glu,[h] pH 5	0.770 (6.21)	2.87 (23.1)	1.60 (55.7)	8.71 (70.2)	
50	38.0	31.1 (82)	Glu,[h] pH 5	1.75 (5.65)	10.1 (32.5)	4.13 (40.9)	19.2 (61.7)	47.2
115	91.0	80.5 (88)	Glu,[h] pH 5	3.88 (4.82)	37.4 (46.5)	9.06 (24.2)	39.2 (48.7)	
202	192	165 (86)	Glu,[h] pH 5	7.66 (4.70)	82.5 (50.0)	20.1 (24.4)	74.8 (45.3)	55.9
28	7.42	7.29 (98)	Glu, pH 6.5	1.74 (23.9)	1.10 (15.1)	0.640 (58.2)	4.45 (61.0)	125
60	37.6	36.0 (95)	Glu, pH 6.5	35.2 (9.78)	8.08 (22.7)	2.67 (33.0)	24.4 (67.8)	125
110	115	104 (90)	Glu, pH 6.5	7.07 (6.80)	27.0 (26.0)	7.78 (28.8)	70.0 (67.3)	126
210	291	252 (86)	Glu, pH 6.5	9.72 (3.86)	91.3 (36.2)	22.8 (25.0)	151 (59.9)	125

[a] A total of ^{59}Fe in initial cell wash and particulate and soluble fractions. [b] Whole cells were washed at 0 °C for 30 min at pH 5.0. [c] Refers to particulate fraction only; washing at 0 °C for 30 min, pH 5. [d] After 60 min of incubation. [e] In nmol/pellet. [f] Percent of total uptake. [g] Ac is acetate-buffered modified Hanks (Kot and Bezkorovainy, 1991). [h] Glu is 3,3-dimethylglutarate buffer (0.1 M); all other components are those of modified Hanks. [i] In percent of recovery. [j] In percent of ^{59}Fe in particulate fraction.

Binding of Iron by the Particulate Fraction of *B. thermophilum*. The particulate fraction of *B. thermophilum*, prepared by the 4-min sonication process, was subjected to iron binding studies at 0 and 37 °C, and typical results are depicted in Figure 2. It will be noted that at both temperatures the uptake (binding) was biphasic, as was observed with whole cells at 0 °C, with a portion of the iron bound rapidly, followed by a slow binding process that continued to increase after 60 min. The amount bound rapidly could be estimated by regression analysis of the time curves (see Table IV), and the results are depicted by Scatchard plots on insets of Figure 2. The parameters calculable from these data were the dissociation constant (K_d) and the maximum number of iron binding sites (B_{max}). These were K_d = 316 and 735 μM at 0 and 37 °C, respectively, and B_{max} = 27.8 and 32.9 nmol/mg of protein at 0 and 37 °C, respectively. At pH 5, therefore, the behaviors of the particulate fractions at 0 and 37 °C were quite similar with the exception of the K_d.

It is noteworthy that the amount of $^{59}Fe^{2+}$ that could be eluted from the particulate fraction depended on outside iron concentration; however, within a specified incubation medium iron concentration, the amount eluted was independent of time (data shown for 60 min only; Table IV) and was very similar to the value calculated by regression analysis. This indicates that a certain amount of iron is bound to the particulate fraction very rapidly,

Table IV. Rapid Binding of $^{59}Fe^{2+}$ to the Particulate Fraction of *B. thermophilum* Determined from Data in Figure 2 and the Amount of $^{59}Fe^{2+}$ Washed Off with 2 mM Cold Fe^{2+}

curve in Figure 2	temp, °C	regression *y*-intercept,[a] nmol of $^{59}Fe^{2+}$/pellet	regression *y*-intercept, nmol of $^{59}Fe^{2+}$/mg of protein	regression line correlation coefficient	washed off with 2 mM cold Fe^{2+},[b] nmol/mg of protein
a	37	1.09	0.97	0.99	0.97
b	37	2.11	1.88	0.99	1.62
c	37	4.09	3.64	0.99	3.99
d	37	7.67	6.82	0.98	7.51
e	0	2.76	2.16	0.98	3.62
f	0	5.07	3.97	0.99	5.64
g	0	8.83	6.92	0.99	7.74
h	0	13.5	10.6	0.75	9.58

[a] For comparison purposes with Table III. [b] 60-min sample only. $^{59}Fe^{2+}$ eluted was independent of incubation time.

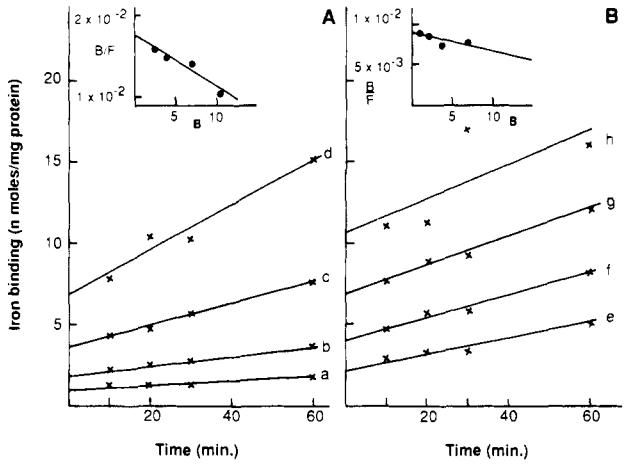

Figure 2. Binding of $^{59}Fe^{2+}$ by *B. thermophilum* particulate fraction at 37 (A) and 0 °C (B). Curves a–h designate iron concentrations of 22, 43, 100, 185, 27, 53, 99, and 201 μM, respectively. Incubation medium was modified Hanks solution at pH 5.0 (Kot and Bezkorovainy, 1991). Lactate production was absent. Inset curves represent Scatchard plots (Scatchard, 1949) using regression values of curves a–d (A) and e–h (B) as per Table V. *B* is bound iron in nanomoles per milligram of protein; *F* is the amount of free $^{59}Fe^{2+}$ in the incubation medium in nanomoles (in a total of 5 mL). It is important to ascertain that no intact cells are present in the particulate fraction preparations.

as previously seen with whole cells at 0 °C, and that this iron is the one that is elutable with cold 2 mM Fe^{2+}.

Binding of $^{59}Fe^{2+}$ by Heated Particulate Fraction of *B. thermophilum*. Particulate fraction of *B. thermophilum* was heated at 80 °C for 15 min and was then subjected to iron binding studies as a function of time with two iron concentrations (100 and 200 μM). The results (data not shown) indicated that the slow binding phase was abolished, while the rapid binding site remained intact. The amount of iron that could be eluted with 2 mM cold Fe^{2+} from the heated particulate fraction was equal to that elutable from the unheated control.

Removal of Iron from $^{59}Fe^{2+}$-Loaded *B. thermophilum*. *B. thermophilum* was loaded with iron in the usual manner, and it was then permitted to incubate in iron-free modified Hanks solution for 60 min at 0 and 37 °C. The release of $^{59}Fe^{2+}$ into the medium was monitored as a function of time, and after 60 min, the cells were sonicated and $^{59}Fe^{2+}$ was determined in the three subcellular compartments. The results are depicted in Table V. At 0 °C, the cells released only 9.63% of recovered iron, whereas 39.5% of the recovered iron was found in the suspension medium at 37 °C. The release of iron from cells was linear over the 60-min period.

The distribution of $^{59}Fe^{2+}$ after the 60-min incubation showed losses from all compartments in the 37 °C batch. However, the loss from the soluble fraction was greater than that from the particulate fraction. The ratios (soluble $^{59}Fe^{2+}$/particulate $^{59}Fe^{2+}$) were 0.61 and 0.86, respectively, for the 37 and the 0 °C experiments. Nevertheless, it is clear that both the particulate and soluble fractions contributed to the $^{59}Fe^{2+}$ lost, and one may, therefore, conclude that an equilibrium exists between soluble and insoluble iron in the cell.

DISCUSSION

The objective of the work described herein was to determine the fate of iron taken up by *B. thermophilum*. Disruption of cells for this purpose was done by sonication for 4 min, which provides for lysis of most, if not all, cells as shown by the lack of lactic acid production. The particulate fraction obtained is assumed to consist of cell wall and cell plasma membrane fragments, with protein/phosphorus ratios of 30–40. Extercate et al. (1970) reported a ratio of 70 for *B. bifidum* var. pennsylvanicus membranes.

When *B. thermophilum* cells were incubated with $^{59}Fe^{2+}$, most of the iron taken up was internalized by an energy-dependent process (Figure 1). Some was bound to the surface of the cells as is indicated by its binding at 0 °C and partial removal with 2 mM cold Fe^{2+}. As cells absorbed more and more iron, lesser percentages thereof were found in the soluble fraction and cell surface, and increasing percentages of iron became associated with the particulate fraction. It could only be partially eluted from the particulate fraction with 2 mM cold Fe^{2+}, this elutable portion being highest when cell iron concentration was low. It may be proposed that most of this particulate fraction-bound iron was bound to the cytosol side of the plasma membrane and that such iron consisted of loosely and tightly bound components. The two compartments may exist in an equilibrium. The iron lost into the iron-free suspension medium when iron-loaded cells were suspended therein came largely from the soluble fraction and, most likely, from the loosely bound fraction associated with the cytosol side of the membrane (see Table V). The above analysis applies to iron distribution achieved at both pH 5.0 and 6.5, except that in the latter case the fraction of iron in the soluble fraction remained fairly constant.

B. thermophilum cells bound iron at 0 °C in a biphasic manner, suggesting two types of bindng sites (see Table I). Iron bound to the rapid binding site was subject to elution by 2 mM cold Fe^{2+}, a conclusion based on the fact that the eluted iron and that calculated by regression analysis showed very similar values. The nature of the slower, heat-sensitive iron binding site remains unknown, although note should be made of the fact that no saturation point was reached after 1 h of incubation time. Also note that the surface-bound iron is some 5–6-fold lower in cells grown in the regular TPY medium as opposed to those grown in the modified TPY medium (Table I), indicating that a low iron growth medium induces the appearance of cell surface iron binding sites.

When the particulate fraction was subjected to iron binding studies, a biphasic uptake behavior was observed as shown in Figure 2. There was not much of a difference between uptakes observed at 37 and 0 °C. It is remarkable that iron binding by the particulate fraction (0 or 37 °C) did not differ much from that of whole cells at 0 °C. Thus, for example, using the nanomoles per pellet expression system, the particulate fraction bound 8.60 and 15.5 nmol of $^{59}Fe^{2+}$/pellet at 37 and 0 °C, respectively, after 60 min, at about 100 μM iron concentration, whereas the cells at the same iron concentration bound 14.3 nmol of iron/pellet (Table I). At about 200 μM iron concentration, the particulate fraction bound 16.9 and 20.5 nmol/pellet, while the cells bound 24.8 nmol/pellet under the same conditions. One may conclude that iron binding by the particulate fraction largely reflects iron binding by the cell surface. At the same time, it may be noted that the particulate fraction was associated with as much as 118 nmol of Fe^{2+}/pellet when it was prepared from iron-preloaded cells (Table III). If we subtract the 20–40 nmol of Fe^{2+} that is associated with the cell surface at 200 mM medium iron concentration, it becomes clear that the cytosol side of the plasma membrane is capable of binding iron and that this activity is lost when the cell is disrupted. The reason for such a loss is yet to be discovered and may involve the

Table V. Exiting of $^{59}Fe^{2+}$ from *B. thermophilum* Suspended in Iron-Free Modified Hanks Solution (Incubation Time, 60 min)

temp, °C	total $^{59}Fe^{2+}$, nmol/pellet	$^{59}Fe^{2+}$ recovered, nmol/pellet	% of recovered $^{59}Fe^{2+}$ located in: supernatant (exiting)	cell surface	cell, soluble fraction	cell, particulate fraction	ratio of soluble to particulate $^{59}Fe^{2+}$	washed off with 2 mM Fe^{2+} from cell particulate fraction, %
37	258	195 (75.6%)	39.5	3.45 (5.68)[a]	21.7 (35.7)	35.5 (58.8)	0.61	16.1
0	258	201 (77.9%)	9.63	5.71 (6.33)	39.0 (43.3)	45.5 (50.4)	0.86	21.6

[a] Values in parentheses are percentages of $^{59}Fe^{2+}$ recovered in the indicated subcellular compartment with respect to total *cellular* iron recovered.

denaturation of a binding protein by sonication, the requirement for a cytoplasmic factor, or another as yet unknown phenomenon characteristic of the intact cell.

It was impossible to subject the data presented in Table I to Scatchard analysis (Scatchard, 1949); however, the particulate fraction lent itself to this approach (Figure 2). It showed that at 0 and 37 °C the number of iron binding sites remained about the same—30 nmol/mg of protein, but that the K_d's were different; the dissociation at 37 °C was easier than it was at 0 °C. Calculation of the maximum number of iron binding sites per bacterial cell, assuming that there are 4.5×10^8 cells/pellet and 1.55 mg of protein/pellet, gave 6.22×10^7 atoms of Fe^{2+}/cell. This represents the rapidly bound iron only. These figures have the limitation that the Scatchard plot was constructed on the basis of only four points each at 0 and 37 °C using single experiments.

It is also remarkable that the amount of $^{59}Fe^{2+}$ eluted from either the particulate fraction or the cells was independent of time and corresponded to the value calculated from regression analysis as per Tables I and Table IV. This indeed confirms the fact that a certain amount of iron is bound to the cell surface very rapidly. It is still unclear whether it is this iron pool or the slower binding iron which then proceeds to become internalized if cells are suspended in a medium at 37 °C in the presence of glucose.

ABBREVIATIONS USED

B., *Bifidobacterium*; TPY, trypticase–phytone–yeast extract; *A*, absorbance; B_{max}, maximum number of binding sites; K_d, dissociation constant.

ACKNOWLEDGMENT

We gratefully acknowledge the technical contribution of Mr. George Haloftis to this work. We thank Dr. Robin Miller-Catchpole for performing bacterial counts and for helpful suggestions and discussions.

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Received for review September 17, 1992. Accepted November 23, 1992.