Iron Accumulation by Bifidobacteria at Low pO_2 and in Air: Action of Putative Ferroxidase

Eva Kot, George Haloftis, and Anatoly Bezkorovainy*

Department of Biochemistry, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612

Bifidobacterium thermophilum (ATCC 25866) and *Bifidobacterium breve* (ATCC 15700) were investigated for their ^{59}Fe accumulation activities in air and at low pO_2 (53 mmHg) using $^{59}Fe^{2+}$. While iron accumulation was only slightly less at low pO_2 than in air, cellular iron distribution showed significant differences: at low pO_2 most cellular iron was soluble Fe^{2+}, whereas in air most iron was precipitated apparently as $Fe(OH)_3$ and associated with the particulate fraction. In the presence of glucose, at pH 6.5, iron could be precipitated in air by the particulate fraction but not by the soluble fraction. Heated particulate fraction accumulated little ^{59}Fe. It is proposed that bifidobacteria contain an intracellular ferroxidase, which catalyzes the oxidation of Fe^{2+} to Fe^{3+} in the presence of O_2. The putative ferroxidase has an apparent K_m of 518 ± 130 μM with respect to Fe^{2+}, it is associated with the particulate fraction of bifidobacteria, its activity is much greater at pH 6.5 than at pH 5.0, and it apparently requires glucose or another similar carbohydrate as a positive effector.

INTRODUCTION

Bifidobacteria are air-tolerant anaerobes that inhabit the intestinal tracts of both human beings and animals (Bezkorovainy and Miller-Catchpole, 1989). They are being used as supplements in various dairy products, especially in Europe and Japan, and are believed to bestow various health benefits upon the consumers thereof (Kurmann and Rasic, 1991).

Bifidobacteria are capable of accumulating large quantities of iron if they are grown in a metal-poor medium and if iron is presented in the ferrous state (Kot and Bezkorovainy, 1991). This ability to sequester iron, both at pH 5 and 6.5, has been related to the nutritional immunity phenomenon (Bezkorovainy and Solberg, 1989). Iron uptake studies have been carried out largely in air, since bifidobacteria survive in air perfectly well and produce lactic acid (Kot and Bezkorovainy, 1993). In fact, O_2 is taken up by several bifidobacterial species as carbohydrates are metabolized (Shimamura et al., 1990). The only iron uptake studies in reduced O_2 atmosphere were done in 1984 at 25 °C, and little if any difference in overall $^{59}Fe^{2+}$ uptake between air and reduced O_2 at atmosphere was observed using *Bifidobacterium bifidium* var. *pennsylvanicus* (Bezkorovainy, 1984).

This paper is concerned with ^{59}Fe accumulation by bifidobacteria in air and at reduced pO_2. The observations reported herein have led us to propose that bifidobacteria possess a ferroxidase which uses O_2 as a substrate and which converts ferrous iron to the ferric state.

MATERIALS AND METHODS

Microorganisms. *Bifidobacterium thermophilum* (*B. thermophilum*) (ATCC 25866) and *Bifidobacterium breve* (*B. breve*) (ATCC 15700) were purchased from American Type Culture Collection (Rockville, MD). On a day-to-day basis, these organisms were propagated in the trypticase-phytone–yeast extract (TPY) medium described by Scardovi (1986). For experimentation purposes, microorganisms grown in the TPY medium were inoculated into 125-mL bottles containing "modified TPY medium" described in detail by Kot and Bezkorovainy (1991). The main difference between it and the TPY medium was that metals were omitted from the former. Unless otherwise stated, all experiments were performed on organisms grown in the modified TPY medium. Quality control methods, counting of the organisms and definition of the "pellet" are described by Kot and Bezkorovainy (1993).

Iron Accumulation Procedures. Methods for measuring the accumulation of $^{59}Fe^{2+}$ have been described (Kot and Bezkorovainy, 1991, 1993). Two media for iron accumulation were used: that buffered at pH 5.0 with 0.1 M acetate or 3,3-dimethylglutarate and that buffered at pH 6.5 with 3,3-dimethylglutarate. Other components in the two solutions contained the following per liter: 0.4 g of KCl, 8 g of NaCl, 0.14 g of $CaCl_2$, and 2 g of glucose (11.1 mM). Occasionally, whenever indicated, the glucose was omitted; the cells in such solutions are termed "nonenergized" (Kot et al., 1993).

Incubations were carried out in Erlenmeyer flasks at 37 or 0 °C in air or at low pO_2. In the latter case, the cell suspensions were first deaerated with a bench-top pump, and then $^{59}Fe^{2+}$ was added while N_2 was being bubbled through the cell suspension. The flasks were then stopped tightly with rubber stoppers and incubated. Sampling during the incubation time was performed while N_2 was bubbled through the suspension. Alternatively, flasks were charged with $^{59}Fe^{2+}$ under N_2 at zero time and not opened until the end of the experiment. pO_2 was determined by a clinical blood gas apparatus. Incubation media exposed to air had a pO_2 of 180 mmHg at both pH 5.0 and 6.5, whereas those that were treated with N_2 had a pO_2 of 53 mmHg at both pH values. The latter is then termed "low O_2 atmosphere" or low pO_2. All iron accumulation experiments were terminated by bringing the samples to 0 °C in an ice bath.

Cell Disruption. Cells were disrupted either by sonication or by the French pressure cell methods. Sonication procedures and partition of cells into the soluble and particulate fractions were described previously (Kot and Bezkorovainy, 1993). The French pressure cell and press were obtained from Aminco (Urbana, IL). It was used at 10 000 lb/in.^{2} pressure. In both cell disruption cases, the extent of disruption was determined in the particulate fraction by measuring lactic acid production over the extent of the experiment. In both cases, no lactic acid production was observed, whereas for intact normal cells, at 37 °C, lactic acid production was between 200 and 300 μg mL^{-1} h^{-1}. Lactic acid production by cells was, of course, nil if glucose was omitted.

Analytical Procedures and Sources of Chemicals. Lactic acid production and protein content of particulate fractions of bifidobacteria were assayed as previously described (Kot and Bezkorovainy, 1993). Measurements of ^{59}Fe were performed in a Gamma 4000 counter (Beckman Instruments, Palo Alto, CA). Visible spectra were determined in a Perkin-Elmer Lambda 2 spectrophotometer (Norwalk, CT).

* Author to whom correspondence should be addressed.

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Table 1. Iron Accumulation by *B. thermophilum* and *B. breve* at Low pO_2 and in Air and in the Presence and Absence of Glucose

organism	[Fe^{2+}] (μM)	atmos	[glucose] (mM)	Fe accum (nmol/pellet) 10 min	30 min	60 min	Fe distrib ratio[a]
B. thermophilum	203	low pO_2	0.0	26.1	37.6	53.1	0.337
	194	low pO_2	11.1	97.2	142	166	0.404
	182	air	0.0	24.9	39.1	64.7	0.561
	182	air	11.1	92.0	159	221	1.551
B. breve	211	low pO_2	0.0	37.2	46.7	41.4	0.268
	196	low pO_2	11.1	70.5	126	135	0.243
	179	air	0.0	46.9	78.8	120	0.844
	190	air	11.1	143	235	312	1.67

[a] This is the ratio of iron recovered in the particulate fraction to that in the soluble fraction after sonication of cells that had been incubated with the indicated amounts of iron from 60 min.

Table 2. Iron Accumulation by *B. thermophilum* and *B. breve* at Low pO_2 as a Function of Iron Content and in the Presence and Absence of Glucose

organism	[Fe^{2+}] (μM)	[glucose] (mM)	Fe accum (nmol/pellet) 10 min	30 min	60 min	Fe distrib ratio[a]
B. thermophilum	45.0	0.0	6.95	8.39	10.6	0.167
	98.0	0.0	18.6	24.5	28.8	0.231
	148	0.0	44.4	56.1	60.1	0.225
	58.0	11.1	19.9	35.1	37.7	0.317
	88.0	11.1	38.9	61.3	72.1	0.285
	144	11.1	63.0	92.2	117	0.419
	191	11.1	89.5	126	142	0.534
B. breve	45.0	0.0	7.18	8.99	9.63	0.215
	91.0	0.0	19.4	22.7	22.2	0.165
	150	0.0	34.8	32.6	34.4	0.332
	198	0.0	47.6	46.8	48.1	0.425
	44.0	11.1	10.7	17.3	21.5	0.317
	97.0	11.1	27.3	44.6	59.6	0.269
	152	11.1	43.1	82.5	124	0.275
	201	11.1	64.6	135	180	0.285

[a] See footnote to Table 1.

All chemicals, including lactic acid determination kits, were purchased from Sigma Chemical Co. (St. Louis, MO). Exceptions were trypticase and peptone, which were obtained from BBL Microbiology Systems (Cockeysville, MD). $^{59}Fe^{2+}$ was obtained from DuPont Laboratories (Boston, MA).

RESULTS

Iron Accumulation by *B. thermophilum* and *B. breve* under Low pO_2 and in Air. Table 1 summarizes iron accumulation data in the 200 μM Fe^{2+} range for *B. thermophilum* and *B. breve* in air and at low pO_2. Addressing ourselves to data obtained in the presence of the glucose only, it will be noted that total iron uptake by *B. thermophilum* at low pO_2 did not differ much from that in air. The difference was, however, much greater in the case of *B. breve*. The most striking difference in both species was observed in the realm of iron distribution: in air, iron recovered in the particulate fraction was much greater than that recovered in the soluble fraction, while the reverse was true at low pO_2.

When these parameters were measured as a function of iron concentration and at low pO_2 (Table 2), it was observed that the amount of iron recovered in the particulate fraction increased as the amount of Fe^{2+} entering the cells increased; however, the ratio of particulate iron to soluble iron remained well below 1. It is well above 1 if the experiments are run in air (Kot and Bezkorovainy, 1993).

Table 3. Iron Accumulation by *B. thermophilum* at Very Low Outside Iron Concentrations at Low pO_2 and in Air[a]

[Fe^{2+}] (μM)	atmos	[glucose] (mM)	Fe accum (nmol/pellet) 10 min	30 min	60 min	Fe distrib ratio[b]
11.2	low pO_2	0.0	0.880	0.970	1.07	0.23
10.9	low pO_2	11.1	2.37	3.11	2.93	0.24
11.3	air	0.0	0.930	1.19	1.52	0.27
11.2	air	11.1	2.43	3.56	3.17	0.26

[a] The medium contained acetate buffer at pH 5.0. [b] See footnote to Table 1.

Table 4. Effect of pH, Glucose, and Atmosphere on the Deposition of Iron on the Particulate Fraction of *B. thermophilum*[a]

cell batch	[Fe^{2+}] (μM)	atmos	[glucose] (mM)	pH	Fe deposition (nmol/mg of protein) 10 min	30 min	60 min
A	181	low pO_2	11.1	6.5	39.8	59.5	70.3
	213	low pO_2	11.1	5.0	20.8	33.5	40.4
B	205	air	11.1	6.5	45.2	84.1	98.2
	223	air	11.1	5.0	16.9	44.7	42.3
C	196	low pO_2	11.1	6.5	47.3	74.4	92.0
	212	low pO_2	0.0	6.5	9.77	12.0	14.4
	222	air	11.1	6.5	53.0	93.0	129
	195	air	0.0	6.5	8.95	12.4	18.7
D	200	low pO_2	11.1	5.0	14.4	24.8	29.1
	209	low pO_2	0.0	5.0	15.67	25.8	29.4
	197	air	11.1	5.0	15.8	26.6	31.2
	200	air	0.0	5.0	14.55	22.0	23.1
E[b]	224	low pO_2	11.1	5.0	18.4	30.0	42.4
	216	air	11.1	5.0	27.7	44.6	68.1

[a] All experiments, except those with batch E, were carried out in the presence of 3,3-dimethylglutarate buffer at 37 °C. [b] In this experiment, the medium was buffered with 0.2 M acetate rather than 3,3-dimethylglutarate.

When iron accumulation experiments were performed at very low iron concentrations, the accumulation of iron was again dependent on the atmosphere; however, the distribution of iron between the particulate and soluble fractions remained the same—in the range 0.23–0.27 (Table 3). The data shown were obtained at iron concentrations of 10.9–11.3 μM, through similar data were obtained for lower iron concentrations: in the [Fe^{2+}] range 1.8–12.7 μM, the particulate fraction iron to soluble iron ratio was 0.24 ± 0.056 (n = 16). Glucose had no effect on the distribution of iron, though total iron accumulation was about twice as great in the presence of glucose (Table 3).

Iron Deposition by Particulate and Soluble Fractions of *B. thermophilum*. Particulate and soluble fractions were prepared either by sonication or by the French pressure cell. The effects of four parameters on iron deposition were studied: glucose, pH, catalase, and atmosphere. We will focus on the effects of pH and atmosphere in this section. Because of rather wide batch-to-batch variations, data in Table 4 are listed for five different particulate fraction preparations. This table shows that more iron is deposited on the particulate fraction of *B. thermophilum* at pH 6.5 than at pH 5.0 in both the air and low O_2 atmospheres. The difference between iron deposited by the particulate fraction in air and at low pO_2 at pH 5.5 is more pronounced in the acetate than the 3,3-dimethylglutarate buffer (Table 4, batches D and E). At pH 6.5, the amount of iron deposited on the particulate fraction in low O_2 atmosphere was 50.8 ± 25.8% (n = 4) of that in air.

When 5 mL of the soluble fraction at pH 6.5 was incubated with 198 μM $^{59}Fe^{2+}$ in air, the following results were obtained after addition of 2 mg of kaolin and

centrifugation: the pellet contained 0.530, 1.01, and 1.19 nmol of iron after 10, 30, and 60 min, respectively. The soluble fraction was prepared via the French pressure cell. Similar results were obtained with the soluble fraction prepared by sonication (data not shown).

The deposition of iron by the particulate fraction in air was dependent on iron concentration and could be described by double-reciprocal plots. The rates of iron deposition were very constant beyond the instantaneous binding of a small amount of iron, so that the rates could be calculated over a broad spectrum of time. Considerable batch-to-batch variations were observed with an overall K_m of 518 ± 130 μM (n = 5) and a V_{max} of 13.47 ± 2.59 nmol (mg of protein)$^{-1}$ min^{-1} (n = 5) with respect to Fe^{2+}.

Deposition of Iron by *B. thermophilum* Particulate Fraction at 0 °C and after Heating. Iron deposition experiments using the *B. thermophilum* particulate fraction were carried out at 0 °C in air and at low pO_2 and in the presence and absence of glucose. The medium was buffered by acetate at pH 5.0. In all cases, at $[Fe^{2+}]$ = 200 μM, iron deposition was almost instantaneous (monophasic), and identical amounts of iron were deposited in air and low pO_2 (8.7 nmol/mg of protein after 60 min). The presence or absence of glucose in the medium gave identical results (data not shown).

As had been discovered previously (Kot and Bezkorovainy, 1993), the heated particulate fraction did not accumulate iron well. This was now demonstrated at both pH 5.0 and 6.5: at iron concentrations of about 217 ± 10 μM and 37 °C, the heated particulate fraction (80 °C for 15 min) accumulated 8.27 and 6.19 nmol of iron/mg of protein at pH 6.5 and 5.0, respectively, after 60 min of incubation. The corresponding unheated materials accumulated 70.9 and 29.6 nmol of iron/mg of protein. Iron accumulation was thus the same for heated particulate fractions at 37 °C and the unheated ones at 0 °C.

Nature of Accumulated Iron. Following iron accumulation experiments by intact bifidobacterial cells or their particulate fractions, these structures were routinely washed with 2 mM nonradioactive $FeSO_4$ solution. If such iron accumulation experiments are performed at 0 °C or with heated particulate fractions, most of the deposited iron can thus be removed (Kot and Bezkorovainy, 1993). It is presumed to be ferrous iron and surface-bound. However, iron accumulated by the particulate fraction at 37 °C, either directly or following cell disruption by sonication, is removed only to 10–25% extent by such washing. Most iron deposited in the particulate fraction apparently remains insoluble. Such iron could be largely extracted from the particulate fraction by 1 M mannitol or sorbitol in 4 M NaOH. In nine experiments, the $FeSO_4$ wash removed 20.6 ± 4.03% of deposited iron, and the sorbitol or mannitol washing solubilized 57.6 ± 5.83% of the accumulated iron for a total recovery of 79 ± 4.15%. Visible spectrum of the sorbitol/mannitol extract containing about 0.9 mM iron indicated absorption maxima at 540 and 650 nm. The latter is characteristic of ferric hydroxide–polyalcohol complexes (Schneider et al., 1982). Sorbitol or mannitol alone showed an absorption maximum at 540 nm only.

The iron recovered in the soluble fraction of sonicated cells was fully chelatable by Chelex 100 (Na form, Bio-Rad Laboratories, Richmond, CA), a cation-exchange resin, and hence not protein-bound. Such supernatant fractions were also ferrozine-positive (data not shown). Ferrozine gives a color with ferrous but not ferric iron.

Effects of Carbohydrates and Catalase. In our previous publication (Kot and Bezkorovainy, 1993), it was reported that *B. thermophilum* particulate fraction was able to precipitate iron in acetate buffer at 37 °C and pH 5 at about the same level as a 0 °C. If glucose is present, however, the accumulation of iron by the particulate fraction rises dramatically. This is especially evident if the pH is 6.5 and if the experiment is done in air (Table 4). At low pO_2 and pH 5, glucose made little if any difference. It should be noted that such particulate fractions, whether prepared by sonication or the French pressure cell, were not active in producing lactic acid, thus eliminating the possibility of intact cell effects.

Several carbohydrates or carbohydrate-like substances were tested as glucose replacements in air at pH 6.5. At the level of 11.1 mM, the carbohydrates had the following ability to aid in depositing iron on the particulate fraction of *B. thermophilum* compared to glucose: galactose, 93%; sucrose, 88%; maltose, 109%; fructose, 56%; mannose, 44%; sorbitol; 35%; ribose, 33%; no carbohydrate, 34%. Sorbitol and ribose thus had no significant activity. The effect of glucose was concentration-dependent.

The possibility exists that the deposition of iron on the particulate fraction in air may be enhanced by a free-radical mechanism. This is usually associated with H_2O_2 production. In fact, the requirement for glucose may be related to its ability to generate H_2O_2 via a putative glucose oxidase reaction. To test for such possibilities, the iron accumulation experiments were performed at pH 6.5 in the presence of 1800 units/mL of mouse liver catalase on both intact *B. thermophilum* cells and its particulate fraction. Catalase has no effect on iron accumulation in either system (data not shown).

Though circumstantial evidence indicates that ferrous iron in the intact cell was being oxidized to the ferric state in the presence of oxygen by an enzymatic process located in the cell interior, the possibility that this process was taking place on the cell exterior could not be rigorously excluded. However, taking advantage of the carbohydrate effects described above, this possibility can be all but eliminated. Thus, if the oxidation and iron deposition process were a cell surface phenomenon, then such carbohydrates as galactose and maltose, which enhanced iron deposition on the particulate fraction of *B. thermophilum*, should act in the same way with intact cells. This, in fact was not the case. At the level of 11.1 mM, at pH 6.5 in air, galactose was completely inactive (identical to the carbohydrate-free system), while maltose was 40.5% as active as glucose in stimulating iron deposition by the cells after 60 min of incubation. Since maltose is a glucose disaccharide, this minor activity may be explained by the presence of small amounts of glucose in the maltose preparation, developed, perhaps, by nonspecific hydrolysis of maltose. It should be noted that the cells used in these experiments were grown in the presence of glucose.

DISCUSSION

The nature of iron deposition by the particulate fraction of bifidobacteria has been somewhat of a puzzle in view of its biphasic nature. Data presented in this paper suggest that we are dealing, on the one head, with a classical iron-binding phenomonon (first, very rapid phase) and an enzyme-dependent process (second, slow phase) on the other. This slow phase was inhibited at 0 °C and by heat. What is the nature of this enzymatic process? Experiments with whole cells at low pO_2 and in air have provided a clue: at low pO_2 most of the internalized iron remains in the soluble fraction in the ferrous form (chelexing experiment, ferrozine reaction), whereas in air most of the iron becomes associated with the particulate fraction and not elutable with Fe^{2+}. This indicates that the bifidobacterial cell may

contain a ferroxidase, which would convert ferrous iron to the ferric form in the presence of O_2. The ferric iron would then precipitate as $Fe(OH)_3$ or its polymer because of its very low solubility product at neutrality. It would then become associated with the particulate fraction of the cell, both in whole cell and in isolated particulate fraction experiments. The iron, which we referred to in the past as being "bound" to the particulate fraction, is in reality not bound but coprecipitated and cocentrifuged as $Fe(OH)_3$ with the particulate fraction. Experiments with the sorbitol extraction indeed suggest that we are dealing with $Fe(OH)_3$.

The putative ferroxidase is apparently associated with the particulate fraction of the cell, since we observed little if any conversion of ferrous iron to its ferric state in the cell soluble fraction. Moreover, the putative enzyme is much more active at pH 6.5 than it is at pH 5. It should be noted that the cell interior pH should also be close to neutrality regardless of the pH of the suspension medium.

Is it possible that in all cases we are observing a nonspecific oxidation of Fe^{2+} to Fe^{3+}? This would be especially relevant to experiments with the particulate fraction, where the slow phase of iron deposition could be explained by its slow oxidation to the ferric state. If that were so, then there should have been no difference observed between the heated and unheated particulate fractions, yet profound differences were observed. Moreover, when 200 μM ferrous iron was incubated at pH 5 and 6.5 for 1 h, the amount of ^{59}Fe that was centrifuged down with the kaolin was less than that observed with the bifidobacterial soluble fraction (data not shown). It may be concluded that little if any nonspecific oxidation of ferrous iron was taking place under our experimental conditions at both pH 5.0 and 6.5.

The presence of a putative ferroxidase in the cell would tend to explain why the uptake of Fe^{2+} is somewhat greater in air than under N_2. In the latter case, most of the internalized iron remains soluble, thus posing a barrier to the entry of additional iron into the cell, especially since this is done by an active process against gradient. But if the iron is precipitated in the form of $Fe(OH)_3$, the gradient becomes greater and pumping of iron into the cell easier. The presence of a ferroxidase could also explain why in iron uptake experiments by whole cells (Bezkorovainy and Solberg, 1989; Kot and Bezkorovainy, 1993), no iron saturation levels could be demonstrated. Iron uptake seemed to proceed in an unlimited fashion. This would, of course, be expected if the accumulated iron were deposited as $Fe(OH)_3$ inside the cell by the action of a ferroxidase, leaving relatively low Fe^{2+} concentrations in the soluble fraction.

The results depicted in Table 3 are interesting in that they indicate that the putative ferroxidase may not be active with very low iron concentrations—in the range 1–12 μM. This may be a reflection of a high K_m of the putative enzyme with respect to Fe^{2+} (500 μM range). On the other hand, its K_m with respect to O_2 may be quite low.

The effects of glucose are quite puzzling. Our data are consistent with the possibility that glucose, and possibly other carbohydrates, act as positive effectors of the putative ferroxidase. It may also be that a substance produced from glucose rather than glucose itself is the actual positive effector, though H_2O_2 has been excluded from this role.

What then is the biological significance of bifidobacterial ferroxidase? Bifidobacteria are gastrointestinal tract organisms in both human beings and animals, and we have proposed that one of their functions may be in the realm of nutritional immunity, i.e., sequentration of iron to keep it away from pathogens. But too much iron may kill the cells, and so there seems to be a system in place that converts soluble iron to the insoluble and relatively innocuous $Fe(OH)_3$. Obviously not much of a pO_2 is required for this reaction, while iron concentration must reach fairly high levels (>40 mM) for the putative ferroxidase to go to work. Thus, the bacteria can perform their assigned function and survive the relatively high iron concentrations that may develop in their soluble fractions.

This work suggests that ferroxidase is present in bacteria, most likely in the cell interior, though the possibility of a ferroxidase on the cell surface has not been rigorously excluded. Nevertheless, ferrous iron appears to be oxidized in the presence of bifidobacterial particulate fraction. This follows Michaelis–Menten kinetics, and the phenomenon is heat-sensitive and does not take place at 0 °C. That this is a complex process is attested to by the role played by glucose. Further information on this putative enzyme will have to await its isolation from bifidobacteria.

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