Ferrous Iron Oxidation by *Lactobacillus acidophilus* and Its Metabolic Products

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Lactobacillus acidophilus (ATCC 4356), grown in trypticase–phytone–yeast extract medium under anaerobic conditions, was capable of accumulating nanomolar quantities of iron when presented with Fe^{2+} at pH 6.5. A major factor in the ability of the organism to accumulate iron was its ability to produce and export H_2O_2, which oxidized Fe^{2+} to Fe(III). The latter was bound to the cell surface by a trypsin-sensitive protein. H_2O_2 production was inhibited by low p_{O_2} and glucose. *L. acidophilus* contained an Fe^{2+} transport mechanism and an intracellular heat-sensitive ferroxidase comparable to that of bifidobacteria. Lactate produced by the organism facilitated Fe^{2+} oxidation by O_2, but the Fe(III) produced remained sequestered by the lactate. It was concluded that *L. acidophilus* may participate in the nutritional immunity scheme under certain circumstances.

Keywords: *Iron; hydrogen peroxide; Lactobacillus acidophilus*

INTRODUCTION

The metabolism of ferrous iron in bifidobacteria has been explored to a considerable extent in our laboratory. It was determined that ferrous iron is internalized in relatively large amounts by these organisms, providing they are grown in a metal-depleted medium, which we termed the modified trypticase–phytone–yeast extract (TPY) medium (Kot and Bezkorovainy, 1991). Though iron uptake by bifidobacteria was energy-dependent, considerable amounts of iron were taken up in the absence of a carbon source as long as the pH of the medium remained well below that of the cell interior (Kot et al., 1993).

In the cell interior, iron was partitioned between the soluble and the particulate fractions, the ratio of which depended on both the iron concentration and the availability of oxygen: the higher the concentration of iron, the lower the soluble/particulate iron ratio; and the less oxygen in the medium, the higher the ratio (Kot and Bezkorovainy, 1993; Kot et al., 1994). Cellular soluble iron was identified as Fe^{2+}, whereas that of the particulate fraction was Fe(III). On the basis of these and other data, it was proposed that bifidobacteria contained intracellular ferroxidase, which was localized in the particulate fraction of the cell (Kot et al., 1994).

The biological significance of iron accumulation by bifidobacteria has been related to the nutritional immunity phenomenon (Bezkorovainy and Solberg, 1989). This phenomenon revolves around the notion that certain iron-binding proteins (and bacteria, as per our proposal) can sequester iron so as to make it unavailable for the proliferation of pathogens (Weinberg, 1986).

There have been numerous suggestions that bifidobacteria and lactic acid bacteria are beneficial to human and animal health throughout their life cycles, and for this reason, bifidobacterial and lactic acid bacterial cultures have been added to various food products (Kurmann and Rasic, 1991; Robinson, 1991). Such bacterial cultures, upon proliferation in the intestinal tract, produce considerable amounts of acetic and/or lactic acid buffers and thus lower the pH of the intestinal contents. This, along with the possible nutritional immunity properties of these organisms, serves to limit the growth of pathogens.

Though ferrous iron uptake and intracellular oxidation of Fe^{2+} to Fe(III) in bifidobacteria are now reasonably well documented, there is very little if any information on this phenomenon in lactic acid bacterial species used in the food industry. Yet such organisms, e.g., the lactobacilli and streptococci, are used much more extensively as food additives than are bifidobacteria, and their health benefits may be just as extensive as, if not more so than, those of bifidobacteria. For this reason, a survey was made of four industrially important lactic acid bacterial species with respect to their ferrous iron-oxidizing abilities (Kot et al., 1995). It was found that *Streptococcus thermophilum* and *Lactobacillus plantarum* were able to accumulate iron in a manner similar to that of bifidobacteria. On the other hand, ferrous iron could be oxidized by H_2O_2 elaborated into the medium by *Lactobacillus acidophilus* and *bulgaricus*. Since *L. acidophilus* is used so widely in various food preparations, it was decided to explore its iron-oxidizing properties to a greater extent, which is the subject of the present paper.

MATERIALS AND METHODS

Microorganisms. *Bifidobacterium thermophilum* (ATCC 25866), *Bifidobacterium breve* (ATCC 15700), and *Lactobacillus acidophilus* (ATCC 4356) were purchased from American Type Culture Collection (Rockville, MD). These organisms were propagated under anaerobic conditions in the trypticase–phytone–yeast extract (TPY) medium (Scardovi, 1986). For experimental purposes, bifidobacteria were inoculated for growth into 125 mL bottles containing the modified TPY medium described by Kot and Bezkorovainy (1991), whereas *L. acidophilus* was inoculated into the normal TPY medium. This organism did not grow well in the metal-depleted modified TPY medium and often gave irreproducible results when tested for iron-accumulating properties. It grew well in the TPY medium, giving a turbidity reading of 1.2 at 610 nm after 18 h of growth.

Trypsin-digested organisms were prepared by incubating cell suspensions, A_{610} = 1.2 in the 0.1 M dimethylglutarate buffer at pH 6.5, with 0.66 mg/mL trypsin and 0.11 mg/mL $CaCl_2$ for 30 min at 37 °C with shaking. The cells were then separated from the digestion medium by centrifugation and

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suspended in a fresh batch of the buffer for iron uptake/binding studies. The amount of trypsin used represents 5% of cellular wet weight.

Iron Accumulation and Oxidation Assays. Iron accumulation assays for cells and their particulate fractions have been described previously (Kot and Bezkorovainy, 1991, 1993; Kot et al., 1994). The 0.1 M 3,3-dimethylglutarate buffer at pH 6.5 was used throughout in the assay media. Occasionally, 0.1 M imidazole buffer at pH 6.5 was also used for buffer effect testing, but no differences were detected. Glucose (2 mg/mL or 11.1 mM) was either present or omitted from the assay medium as indicated. Note should be taken that, unless otherwise indicated, stock solutions of the 10 mM $^{59}Fe^{2+}$ contained 5.7 mM ascorbate as an antioxidant, since ferrous iron is easily oxidized to the ferric form [Fe(III)] at neutral pH.

All iron accumulation data by cells were expressed in terms of nanomoles of iron per bacterial pellet (Kot and Bezkorovainy, 1993). A pellet represents that number of microorganisms which is present in 5 mL of a bacterial suspension with an $A_{610} = 1.2$. When incubated in the presence of 2 mg/mL glucose (total of 10 mg of glucose), a pellet metabolized 90% of the glucose in 60 min at 37 °C. Accumulated iron is the sum total of iron incorporated into the cell interior, iron bound to the cell surface as Fe^{2+}, and, under some circumstances, Fe(III) formed extracellularly that becomes associated with the cells. Where indicated, surface-bound Fe^{2+} was estimated by washing the cells with cold 2 mM Fe^{2+} (Kot et al., 1993). The term "pellet equivalent" (also 5 mL in volume) was used when the supernatant of a pellet was tested for its Fe^{2+} oxidizing ability.

Fe(III) solutions (these may have been colloidal suspensions) used to assess Fe(III) binding by bacteria and their particulate fractions were prepared as follows: To 100 mL of the 0.1 M dimethylglutarate buffer at pH 6.5 was added 2 μL of 30% H_2O_2. After mixing, 2 mL of the 10 mM $^{59}Fe^{2+}$ stock solution was added to make an approximately 200 μM ^{59}Fe(III) solution. It immediately assumed a typical amber coloration. Dilution with the pH 6.5 buffer was made if Fe(III) concentrations of less than 200 μM were desired.

The assay for Fe^{2+} oxidation to Fe(III) in cell-free solutions (pellet equivalents) was done by measuring Fe^{2+} concentrations with ferrozine before and after the reaction. Ferrozine gives a color with ferrous iron only, so the difference represented Fe(III) formed or Fe^{2+} oxidized. The ferrozine assay was based on a procedure developed by the Sigma Corp. (St. Louis, MO) and marketed as serum iron analysis kit 565: 0.5 mL of the sample was mixed with 2 mL of 0.1 M acetate buffer at pH 5.0, and then 0.05 mL of 0.85% ferrozine in the acetate buffer was added. The sample was incubated at 37 °C for 10 min and then read at 560 nm. $FeSO_4$ solutions served as standards. This method was not sensitive below $[Fe^{2+}] = 50$ μM. Iron accumulation studies at low p_{O_2} (53 mmHg) were done as previously described (Kot et al., 1994).

Cell Disruption. Bacterial cells were disrupted by sonication or the French pressure cell method as previously described (Kot and Bezkorovainy, 1993). This was done for the purpose of partitioning iron into the insoluble [particulate fraction, Fe(III)] and soluble (Fe^{2+}) fractions. In addition, the particulate fractions of bacteria were also tested for their Fe^{2+}-oxidizing activity and hence the presence of a putative ferroxidase (Kot et al., 1994). Neither the soluble nor the particulate fractions were able to generate much lactic acid.

Chemicals and Analytical Procedures. Most chemicals and analytical reagents were purchased from Sigma. Trypticase and peptone, used for the preparation of bacterial growth media, were purchased from BBL Microbiology Systems (Cockeysville, MD). Radioactive Fe^{2+} was obtained from DuPont Laboratories (Boston, MA).

Ultraviolet light and visible light absorption spectra were measured in a Perkin-Elmer Lambda 2 spectrophotometer (Norwalk, CT). $^{59}Fe^{2+}$ was counted in a Gamma 4000 counter (Beckman Instruments, Palo Alto, CA).

Hydrogen peroxide was assayed with a Sigma glucose oxidase kit 510, which is designed to measure blood glucose concentrations and uses *o*-dianisidine as a reducing agent, and with cholesterol determination kit 352, which uses a quinoneimine dye as a reducing agent. The latter, but not the former, could be used in the presence of glucose. These kits were not sufficiently sensitive to reliably determine H_2O_2 concentrations of below 100 μM.

L-Lactic acid was measured by Sigma kit 826 UV. This kit was able to detect lactic acid produced by all organisms studied herein, since they are L(+)-lactate producers. Protein determination was done according to the Lowry method (Lowry et al., 1951). It was used to express the degree of ferrous iron oxidation by bacterial particulate fractions.

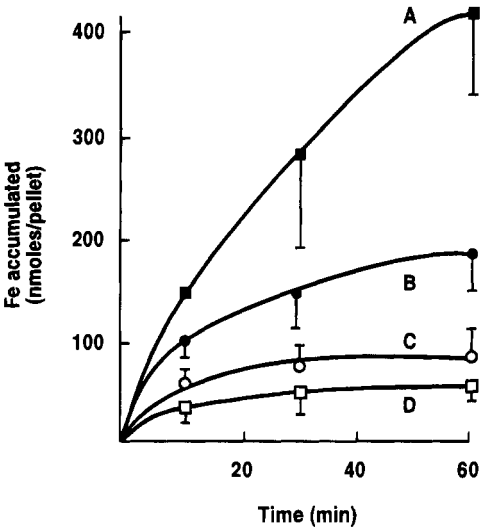

Figure 1. Iron accumulation by *L. acidophilus* at 37 and 0 °C and in the presence and absence of glucose. All experiments were done in air in the pH 6.5 3,3-dimethylglutarate buffer. (curve A) No glucose, 37 °C, $[Fe^{2+}] = 209 \pm 14.6$ μM, lactate (60 min) = 42.1 ± 12.9 μg/mL, 2 mM Fe^{2+} wash (60 min) = 26 ± 2.1% of accumulated iron; (curve B) 11.1 mM glucose, 37 °C, $[Fe^{2+}] = 193 \pm 13.4$ μM, lactate (60 min) = 754 ± 112 μg/mL, 2 mM Fe^{2+} wash (60 min) = 17.4 ± 3.4% of accumulated iron; (curve C) 11.1 mM glucose, 0 °C, $[Fe^{2+}] = 194 \pm 3.90$ μM, lactate (60 min) = 68.6 ± 16.0 μg/mL, 2 mM Fe^{2+} wash (60 min) = 35 ± 3.3% of accumulated iron; (curve D) no glucose, 0 °C, $[Fe^{2+}] = 202 \pm 18.4$ μM, lactate (60 min) = 62.2 ± 14.3 μg/mL, 2 mM Fe^{2+} wash (60 min) = 38 ± 9.7% of accumulated iron.

RESULTS

Iron Accumulation by *L. acidophilus*. Iron accumulation studies by *L. acidophilus* with $^{59}Fe^{2+}$ in the medium were carried out at pH 6.5. Figure 1 illustrates iron accumulation as a function of time in the presence of approximately 200 μM Fe^{2+} with and without glucose at 37 and 0 °C. Considerable batch-to-batch variation was observed, which is reflected in the high standard deviations indicated. When the cells incubated at 37 °C were washed with 2 mM $FeSO_4$, those incubated in the presence of glucose lost 17 ± 3.4% of accumulated iron, while those incubated without glucose lost 26 ± 2.1%. This iron is representative of loosely bound surface iron.

As discovered previously (Kot et al., 1995), more iron became associated with the cells in the absence of glucose than in its presence at 37 °C. This is the reverse of what happens with bifidobacteria at pH 6.5 (Kot et al., 1993). At 0 °C, on the other hand, the two values were approximately the same, and the percentage of iron lost after washing with 2 mM Fe^{2+} was much higher at 0 °C than than at 37 °C (Figure 1). Lactate determinations after 60 min of incubation at 0 or 37 °C in the absence of glucose showed only background values.

Table 1. Iron Accumulation by *L. acidophilus* as a Function of [$^{59}Fe^{2+}$] and in the Presence and Absence of Glucose[a]

[$^{59}Fe^{2+}$] (μM)	glucose (11.1 mM)	^{59}Fe accum (nmol/ pellet)	% washed off with 2 mM Fe^{2+}	ratio of particulate to soluble ^{59}Fe in pellet	lactate prod (μg/mL)
56.0	yes	53.0	13	5.7	578
97.0	yes	120	13	4.6	541
161	yes	154	16	5.4	539
200	yes	183	15	4.1	553
28.0	no	44.2	22	3.2	35
54.0	no	97.4	20	3.7	34
111	no	246	20	4.0	33
214	no	363	28	3.9	36

[a] All incubations were carried out at 37 °C for 60 min in the pH 6.5 dimethylglutarate buffer in air.

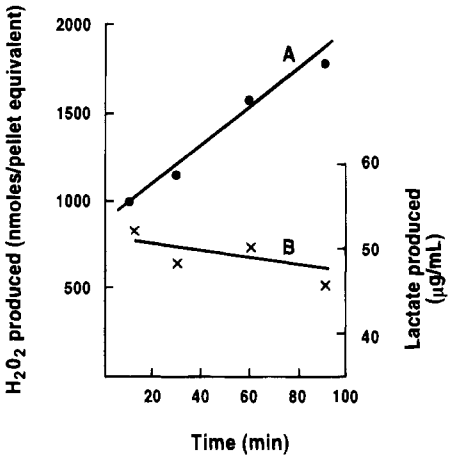

Figure 2. Hydrogen peroxide (curve A) and lactate (curve B) production in air by *L. acidophilus* as a function of time. Hydrogen peroxide was assayed via the *o*-dianisidine reaction. Incubation medium was 0.1 M 3,3-dimethylglutarate buffer at pH 6.5, temperature was 37 °C, and no glucose was added. The 30 min sample contained 240 μM H_2O_2.

The amount of iron accumulated by *L. acidophilus* in the presence and absence of glucose was concentration-dependent as shown in Table 1. Again, at equivalent outside Fe^{2+} concentrations, more iron became associated with the cells if they were incubated without glucose than with glucose. An additional feature shown in this table is the ratio of insoluble to soluble iron following cell disruption. These figures did not vary much with total cellular iron concentration and were considerably higher than those observed in bifidobacteria (Kot and Bezkorovainy, 1993). However, glucose in the medium made a difference: When iron was taken up in its presence, the insoluble/soluble iron ratio was 4.8 ± 0.66 ($n = 10$), whereas in the absence of glucose, it was 3.3 ± 0.58 ($n = 8$), $p < 0.0001$.

Oxidation of Fe^{2+} by *L. acidophilus* Is, in Part, Due to the Release of Hydrogen Peroxide. It is well established that some lactobacilli, including *L. acidophilus*, may elaborate hydrogen peroxide into the medium in the presence of O_2 (Condon, 1987). Our laboratory has further shown that at pH 6.5 in 0.1 M dimethylglutarate buffer this happens only in the absence of glucose (Kot et al., 1995). Production of hydrogen peroxide and lactate in the absence of glucose is shown in Figure 2. It is seen that H_2O_2 release is biphasic, with a rapid release taking place within 10 min, followed by a slower phase that did not abate by 90 min of incubation. For the purpose of future work, the 30 min sample was arbitrarily chosen. The concentration of hydrogen peroxide in such 30 min cell supernatant preparations was 313 ± 128 μM ($n = 12$), the range being between 140 and 660 μM. Lactate remained constant during the experiment.

Table 2 indicates that, as expected, *L. acidophilus* supernatants were able to oxidize Fe^{2+}. The reaction was almost instantaneous and quantitative, i.e., with Fe^{2+} concentrations of 96–191 μM, little if any Fe^{2+} was left, given a 270 μM H_2O_2 concentration. The H_2O_2/Fe(III) ratios were close to 1, as predicted by the Fenton reaction (Yu, 1994), as long as the H_2O_2 concentration was equal to or in excess of that of Fe^{2+}. It should be noted that in these experiments no ascorbate was included in the $^{59}Fe^{2+}$ stock solutions used, as it usually is with cellular iron uptake studies. Had it been included, the oxidation reaction may have been much slower and the H_2O_2/Fe(III) ratio higher. When similar experiments were performed on cell supernatants of *B. thermophilum* and *B. breve*, no oxidation of Fe^{2+} was noted.

Several other experiments were performed with *L. acidophilus* cell supernatants to show that H_2O_2 was indeed responsible for Fe^{2+} oxidation. These involved the treatment of such supernatants with catalase and attempts to release H_2O_2 from the cells at low p_{O_2}. Table 3 presents the results of both sets of experiments. It is clear that catalase abolished the ability of cell supernatants to oxidize ferrous iron and that cell supernatants obtained at low p_{O_2} had a much lower ability to oxidize Fe^{2+}. When cell supernatants obtained at low p_{O_2} were shaken in air for 60 min at 37 °C and then tested for their iron-oxidizing activity, none was observed. This indicates that H_2O_2 was produced intracellularly rather than by some reduced coenzyme (e.g., NADH) released into the medium by the cells.

Figure 1 indicated that considerable amounts of iron became associated with *L. acidophilus* even at 0 °C. The supernatant of cells maintained at 0 °C for 30 min in the absence of glucose was tested for the presence of H_2O_2. While cells kept at 37 °C produced 280 μM H_2O_2, the same cells at 0 °C produced 140 μM H_2O_2. This finding is at odds with that of Collins and Aramaki (1980), who found little if any H_2O_2 production by their strains of *L. acidophilus* at 0 °C.

The presence of glucose in the preincubation medium substantially inhibited the release of H_2O_2 into the medium as shown in Figure 3. The highest iron-oxidizing activity was present in media containing no glucose whatever, and such cells, when subsequently incubated with Fe^{2+} in the absence of glucose, took up (or oxidized) only small amounts of iron. As glucose concentration increased, iron oxidation by cell supernatants declined, and these cells, when subsequently incubated with Fe^{2+} in the absence of glucose, were able to oxidize substantial amounts of Fe^{2+}. It is, however, possible that the presence of glucose itself inhibited Fe^{2+} oxidation by cell supernatants. To examine this possibility, a cell-free supernatant with Fe^{2+}-oxidizing activity was assayed for production of Fe(III) in the presence of 0–5 mg/mL glucose. The level of iron oxidation was identical for all glucose concentrations used (data not shown), indicating that glucose did not inhibit Fe^{2+} oxidation by H_2O_2.

Cell supernatants containing H_2O_2 contained other substances elaborated by *L. acidophilus*, which could be detected by ultraviolet light spectroscopy. There was one compound, apparently a coenzyme, with an absorption maximum at 265 nm. Absorbances ranged from 0.4 to 1 depending on the batch used. There seemed to

Table 2. Oxidation of Fe^{2+} by H_2O_2 Produced by *L. acidophilus* as a Function of Time [in Nanomoles of Fe(III) Produced per 5 mL][a]

total Fe^{2+} initially present (nmol/5 mL)	Fe^{2+} oxidized and H_2O_2 used/Fe^{2+} oxidized ratio							
	2 min		5 min		10 min		60 min	
	Fe(III)	ratio	Fe(III)	ratio	Fe(III)	ratio	Fe(III)	ratio
1465	1105	1.22	1105	1.22	1115	1.21	1210	1.12
955	920	1.47	925	1.46	925	1.46	935	1.44
620	600	1.83	620	1.71	620	1.71	620	1.90
480	480	1.77	480	1.67	480	1.71	480	1.86

[a] Hydrogen peroxide concentration was 270 μM (total of 1350 nmol/5 mL). The 10 mM Fe^{2+} stock solution was freshly prepared in the pH 6.5 3,3-dimethylglutarate buffer without the addition of ascorbate or any other antioxidant.

Table 3. Oxidation of Fe^{2+} by *L. acidophilus* Cell Supernatants: Effect of Catalase and Supernatants Produced at Low p_{O_2}[a]

series	Fe^{2+} used		cell supernatant	Fe^{2+} oxidized[b] (in % of initial Fe^{2+})
	μM	total (nmol)		
A[c]	69.0	345	normal	97
	188	940	normal	85
	296	1480	normal	57
	106	530	with catalase[d]	0.0
	193	965	with catalase	6.0
	290	1450	with catalase	8.0
	279	1395	none: in 5 mL of buffer[e]	11
B	104	520	normal (in air)[c]	98
	194	985	normal (in air)	76
	276	1375	normal (in air)	56
	117	585	obtained at low p_{O_2}[f]	7.0
	205	1025	obtained at low p_{O_2}	9.0
	304	1520	obtained at low p_{O_2}	12

[a] All incubations with iron were for 10 min at 37 °C in air without antioxidants. [b] Fe^{2+} determined by the ferrozine reaction. [c] Cell supernatants contained 220 μM H_2O_2 (1100 nmol/5 mL). [d] Cell supernatants were incubated with 34 000 units of catalase for 30 min at 37 °C before $^{59}Fe^{2+}$ was added. [e] Fe^{2+} incubated in buffer for 10 min at 37 °C. [f] p_{O_2} was 53 mmHg (Kot et al., 1994). There was no detectable H_2O_2.

be no correlation between the H_2O_2 content and absorption at 265 nm. Another well-defined absorption maximum was ascribed to lactic acid. This band, initially at 235 nm, shifted to 240 nm as Fe^{2+} was being slowly oxidized to Fe(III). The shifting was accompanied by an increase in absorbance.

Ferric Iron Binding by *L. acidophilus*. Figure 1 and Table 1 indicate that H_2O_2-oxidized iron was, at least in part associated with the cells. The question arose as to whether the relatively insoluble Fe(III) was simply cocentrifuged down with the cells or was bound specifically or nonspecifically by the cell surface. Fe(III) itself, produced either by *L. acidophilus*-generated H_2O_2 or by H_2O_2 from a reagent bottle, did did not sediment in a centrifuge, nor was any substantial amount brought down by such inert carriers as powdered glass. *L. acidophilus* was, therefore, incubated with H_2O_2-generated $^{59}Fe(III)$ (from $^{59}Fe^{2+}$) as a function of time with the results being depicted in Figure 4. This shows that binding of Fe(III) was almost instantaneous and that it was much smaller at 0 °C than at 37 °C. There was considerable batch-to-batch variation: six experiments gave binding of 121 ± 67.6 nmol of Fe(III)/pellet at Fe(III) concentration of 177 ± 17.2 μM.

Binding of Fe(III) was concentration-dependent as shown in Table 4. Another finding was that trypsin digestion of the cells reduced their ability to bind Fe(III) (Figure 4). Note that the trypsin-digested cells produced lactate at the same rate as normal cells. The particulate fraction of *L. acidophilus* was also able to bind Fe(III) (i.e., H_2O_2-oxidized $^{59}Fe^{2+}$), and this too was diminished by trypsin digestion to 39.7 ± 3.55% of the undigested particulate fraction at 37 °C.

Lastly, the Fe(III)-binding experiments were done in the presence of glucose. In the absence of glucose, *L. acidophilus* was able to bind larger amounts of Fe(III). Thus, in four experiments, the binding of Fe(III) in the presence of glucose was 68.6 ± 18.7% that in its absence. The reason for this is not clear.

Lactate Facilitates Oxidation of Ferrous Iron at pH 6.5. Compared to bifidobacteria, lactic acid bacteria produce enormous amounts of lactic acid. The question, therefore, arises whether or not lactic acid contributes to iron accumulation by *L. acidophilus*. Initial experiments to address this issue revolved around incubating lactate with Fe^{2+} and then measuring the Fe^{2+} remaining as a function of time, lactate concentration, pH, and p_{O_2}. The results of such experiments are shown in Figure 5. This indicates that lactate induced a slow oxidation of Fe^{2+} at pH 6.5, that the extent of oxidation depended on lactate concentration, and that at low p_{O_2} no loss of Fe^{2+} was observed. In addition, it is clear that loss of Fe^{2+} does not occur at pH 5.0.

Lactate also inhibits the accumulation of Fe^{2+} by *L. acidophilus* in the presence of glucose. It will be recalled that little if any H_2O_2 is produced by *L. acidophilus* in the presence of glucose, and any iron accumulation under these circumstances may be attributed to its internalization and subsequent oxidation (see below). As Table 5 indicates, this process is impeded if lactate concentration in such incubation systems is made initially high through the exogenous addition of lactate. It is possible that a lactate–Fe^{2+} complex is formed, making Fe^{2+} unavailable for internalization.

Fe(III) prepared from $^{59}Fe^{2+}$ via lactate-facilitated oxidation was tested for binding with *L. acidophilus*. Only insignificant quantities were bound compared to those seen with H_2O_2-generated Fe(III) (data not shown). This may indicate that the Fe(III) produced remains coordinated with lactate and unavailable for binding.

Internalization and Intracellular Oxidation of Fe^{2+} by *L. acidophilus*. Bifidobacteria were shown to incorporate ferrous iron into their cell interiors and to oxidize it via a putative ferroxidase. A portion of the internalized iron remained in the soluble ferrous form (Kot and Bezkorovainy, 1993). In *L. acidophilus*, this analysis is complicated by the hydrogen peroxide release. However, hydrogen peroxide is not released in the presence of glucose, yet iron accumulation takes place as indicated by Figure 1. If this represents internalization of Fe^{2+}, then is such internalized iron oxidized by intracellular ferroxidases as it is in bifidobacteria? This was explored by performing iron accumulation experiments in the presence of glucose at low p_{O_2} and 37 °C for periods of 15–60 min. Iron accumulation under these conditions was 25.4 ± 14.6%

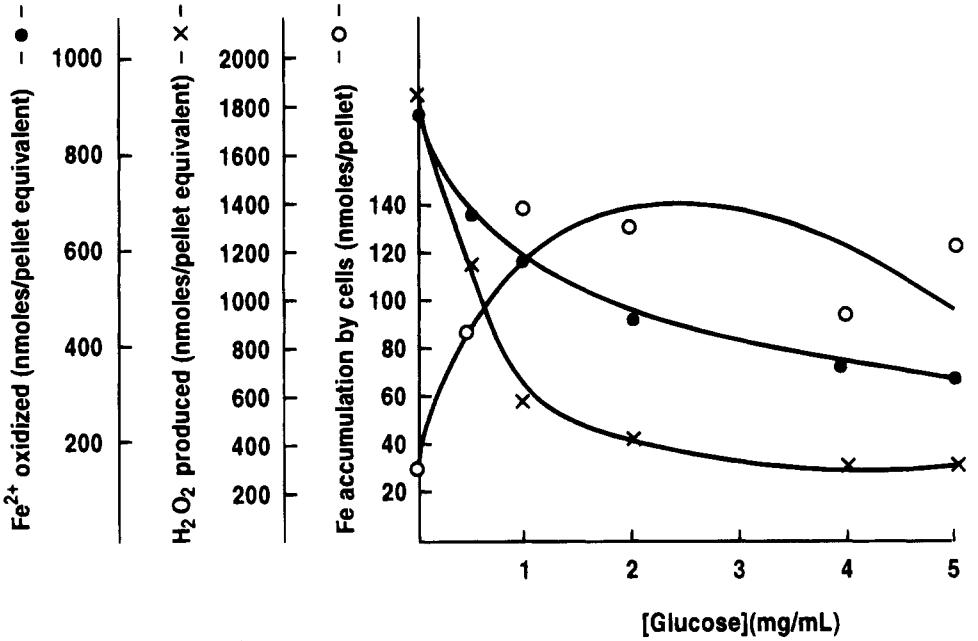

Figure 3. Effect of glucose on the production of hydrogen peroxide by *L. acidophilus*, its ability to oxidize Fe^{2+}, and the capacity of the residual cells to accumulate iron. Cells were incubated in solutions containing various amounts of glucose at pH 6.5 at 37 °C for 30 min in air and then centrifuged. The cells were then subjected to iron accumulation studies in the absence of glucose (○). The Fe^{2+} solutions used contained ascorbate. The cells were incubated at 37 °C in air for 60 min with $[Fe^{2+}] = 217 \pm 8.75$ μM. Iron oxidation by the cell supernatants (●) was carried out at 37 °C in air for 10 min in a total volume of 5 mL (pellet equivalent) with $[Fe^{2+}] = 216 \pm 8.22$ μM. Values shown have been corrected for nonspecific Fe^{2+} oxidation, which was considerable (17% of Fe^{2+} present in 10 min) due to the omission of ascorbate. Production of hydrogen peroxide (×) was measured via the *o*-dianisidine method (in the absence of glucose) and with quinoneimine (in the presence of glucose).

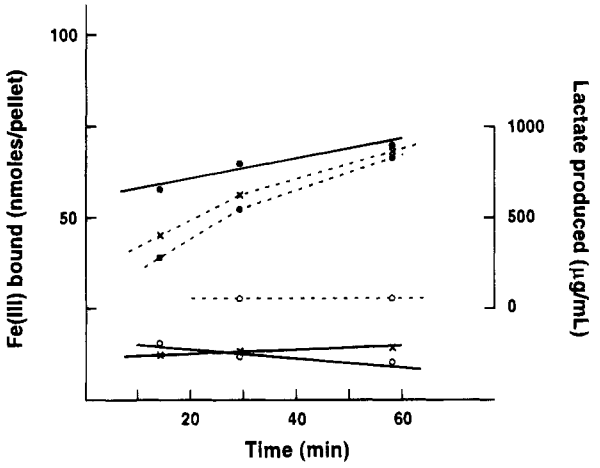

Figure 4. Binding of Fe(III) and lactate production by *L. acidophilus* as a function of time, temperature, and trypsin digestion at $[Fe(III)] = 175$ μM (H_2O_2 generated). Solid circles indicate binding at 37 °C by intact cells; open circles indicate binding at 0 °C by intact cells; and crosses indicate binding at 37 °C by trypsin-digested cells. Broken lines indicate lactate production using the respective symbols.

that in the air at $[Fe^{2+}] = 200$ μM. The insoluble/soluble iron ratio following cell disruption was 1.1 at low p_{O_2} and 4.1 in air in one experiment. This relationship was also observed in bifidobacteria (Kot et al., 1994).

Another experiment to confirm Fe^{2+} transfer into the cell interior was done as follows: Cells were preincubated with and without glucose, centrifuged down, and washed, and then $^{59}Fe^{2+}$ uptake experiments were performed on them in the presence and absence of glucose. The details and results of such experiments are shown in Table 6. The most telling finding was that when cells preincubated without glucose were subjected to these studies, there was little if any iron accumulation in the absence of glucose, indicating that no H_2O_2 was released into the medium. Yet in the presence of glucose, such cells were able to take up iron, indicating that a non-H_2O_2 iron uptake system was in place. Cells preincubated with glucose were able to accumulate iron both in the presence and in the absence of glucose.

Table 4. Concentration Dependence of Fe(III) Binding and Lactate Production by *L. acidophilus*[a]

Fe(III) concn (μM)	Fe(III) bound (nmol/pellet)	lactate prod (μg/mL)
20.0	19.8	872
36.0	42.2	998
67.0	80.7	866
145	142	757

[a] All incubations were performed in air at 37 °C in 0.1 M dimethylglutarate buffer at pH 6.5 in the presence of 2 mg/mL glucose with Fe(III) generated by hydrogen peroxide. Time of incubation was 60 min.

Having established that iron accumulation by *L. acidophilus* may take place by two different mechanisms, i.e., by Fe^{2+} internalization and the surface binding of H_2O_2-generated Fe(III), it becomes clear that the two processes may be taking place at the same time, and the question may be raised as to the relative contribution of each system to the overall iron accumulated. An attempt was made to estimate this as follows: Cells were incubated at 37 °C for 60 min in air in the absence of glucose (as per Table 6) to release the H_2O_2; then both the cells and the supernatant were tested for iron accumulation/oxidation activity. Iron oxidation by the supernatant accounted for about two-thirds of the overall iron accumulated, while cellular internalization of Fe^{2+} accounted for about one-third.

It was noted above that the binding of Fe(III) by *L. acidophilus* was trypsin-sensitive. The uptake of Fe^{2+} by the cells was also diminished by the treatment of cells by trypsin: Trypsin-treated cells took up 35.1 ± 4.58% of the iron taken up by undigested cells ($n = 3$). Such

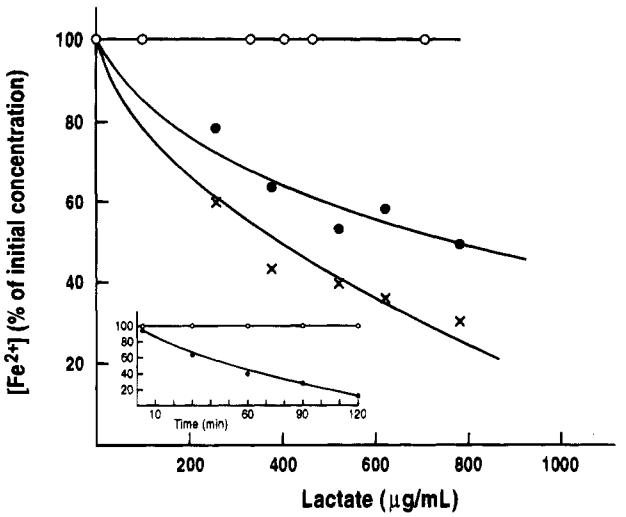

Figure 5. Oxidation of Fe^{2+} in the presence of lactate as a function of time, lactate concentration, and pH at 37 °C in the 0.1 M dimethylglutarate buffer. Crosses indicate a 60 min incubation period at pH 6.5; solid circles indicate a 35 min incubation time at pH 6.5; and open circles indicate a 60 min incubation period at pH 5.0. Temperature was 37 °C, and initial Fe^{2+} concentration was 200 μM. (Inset) Oxidation of Fe^{2+} as a function of time in air (solid circles) and at low p_{O_2} (open circles) at 37 °C, pH 6.5, in the presence of 320 μg/mL lactate with an initial Fe^{2+} concentration of 100 μM.

Table 5. Accumulation of Iron by *L. acidophilus* as a Function of Time and Initial Lactate Concentration[a]

exogenous lactate present at 0 time (μg/mL)	time (min)	total lactate present (μg/mL)	iron accum (nmol/pellet)
250	5	664(448)[b]	36(88)[c]
	10	709(512)	41(76)
	20	811(696)	53(84)
	30	871(795)	59(84)
465	5	699(394)	32(48)
	10	773(473)	38(43)
	30	808(703)	47(43)
	60	844(818)	42(35)

[a] All incubations were performed in air at 37 °C in 0.1 M dimethylglutarate buffer at pH 6.5 at an initial $[Fe^{2+}] = 200$ μM, in the presence of 2 mg/mL glucose. [b] Parenthetical figure is control (no lactate added exogenously). [c] Parenthetical figure is percent of control.

cells produced lactate normally and grew normally under ordinary growth conditions in the TPY medium.

In bifidobacteria, it was proposed that much of the intracellular iron was oxidized by putative ferroxidase(s) associated with their particulate fractions. Particulate fractions, in fact, had ferroxidase activity, where the resulting Fe(III) remained bound to them. Similar experiments were done on *L. acidophilus* particulate fraction, with the results being given in Table 7. Iron was bound by the particulate fraction in a concentration-dependent manner. A considerable amount could be eluted with 2 mM Fe^{2+}. Iron remaining with the particulate fraction may be considered as having been oxidized (Kot et al., 1994). As was the case with bifidobacteria, glucose facilitated the oxidation process; in the absence of glucose, the amount of Fe^{2+} oxidized was 53.8 ± 26.3% of that in its presence ($n = 4$). When heated, the particulate fraction was able to oxidize only 16% of the Fe^{2+} oxidized by the control. Heating was carried out at 80 °C for 15 min. Under low p_{O_2}, oxidation of Fe^{2+} was 50% of that in the air. Trypsin digestion had no effect on the ability of the particulate fraction to oxidize iron (100 ± 15.3%, $n = 3$). Lastly, the oxidative activity of the particulate fraction was somewhat lower than the iron uptake activity of a comparable number of intact cells in the presence of glucose (absence of H_2O_2): 50.4 ± 19.3% for incubation times ranging from 15 to 60 min at 37 °C in air at pH 6.5. Particulate fractions of *L. acidophilus* produced minimal quantities of lactate (Table 7) and produced no detectable hydrogen peroxide.

DISCUSSION

L. acidophilus, a homofermentative air-tolerant anaerobe (Axelsson, 1993), is capable of accumulating considerable amounts of iron when presented with micromolar quantities of Fe^{2+} at pH 6.5. This is accomplished by two mechanisms: internalization of Fe^{2+} followed by intracellular oxidation and extracellular oxidation of Fe^{2+} by H_2O_2 released by the organism followed by surface binding of the Fe(III) produced. This Fe(III) is most likely in the form of the sparsely soluble $Fe(OH)_3$, though that cannot be stated at this time with any degree of certainty. Lactate produced by the bacteria facilitates the oxidation of Fe^{2+} and apparently sequesters the Fe(III) produced, thus diminishing iron accumulation by the bacteria. All of the oxidative processes depend on the availability of O_2.

Lactobacilli are known to produce hydrogen peroxide in the presence of O_2 by a number of mechanisms (Condon, 1987), and it persists because lactobacilli lack catalase (Kandler and Weiss, 1986; Davidson and Hoover, 1993). In *L. acidophilus*, the enzyme NADH oxidase with NADH as substrate has been implicated as the origin of H_2O_2 (Collins and Aramaki, 1980). One may speculate that this is necessary to regenerate NAD from NADH to operate the glycolytic pathway when lactate dehydrogenase is inactive. H_2O_2 production and, by implication, the activity of NADH oxidase were most prominent in the absence of glucose at pH 6.5, this being so presumably because in the absence of glucose the lactate dehydrogenase is inactive. The question that remains, however, is this: What gives rise to NADH,

Table 6. Iron Accumulation by *L. acidophilus*, Which Had Been Preincubated in the Presence and Absence of Glucose[a,b]

glucose in the preincubation medium	cells incubated with glucose		cells incubated without glucose		control cells without glucose[c]	
	iron accum	lactate prod[d]	iron accum	lactate prod[d]	iron accum	lactate prod[d]
yes	77.9 ± 20.7	60.8 ± 10.5	123 ± 19.1	4.87 ± 0.603	179 ± 16.0	7.40 ± 2.19
no	86.5 ± 4.24	84.9 ± 7.93	19.8 ± 13.0	3.50 ± 1.33	174 ± 42.4	6.05 ± 2.04

[a] Preincubations were carried out at 37 °C for 30 min with and without 11.1 mM glucose in the pH 6.5 dimethylglutarate buffer in air. Cells were separated from the preincubation medium and suspended in fresh medium for iron accumulation studies. [b] Iron accumulations are expressed as percent of values observed in nonpreincubated control cells, which were kept on ice during the preincubation process and then subjected to iron accumulation studies in the presence of glucose. Iron accumulation was 198 ± 45.1 nmol/pellet after 60 min of incubation at 37 °C. Lactate production was 795 ± 62.1 μg/mL after 60 min. $^{59}Fe^{2+}$ concentrations were 202 ± 15.0 μM. [c] These cells were kept on ice during the preincubation period and then subjected to iron uptake assays in the absence of glucose. As expected, they accumulated more iron than cells with glucose. [d] In percent of lactate produced by the control cells (see footnote *b*).

Table 7. Oxidation of Ferrous Iron by the Particulate Fraction of *L. acidophilus* as a Function of [Fe^{2+}][a]

[$^{59}Fe^{2+}$] (μM)	Fe^{2+} oxidized		% washed off by 2 mM $FeSO_4$	lactate prod (μg/mL)
	nmol/ pellet	nmol/mg of protein		
24.0	43.8	42.8	19	50
42.0	86.6	84.5	25	47
89.0	112	109	29	48
170	132	129	27	59

[a] Incubations were carried out for 60 min at 37 °C at pH 6.5 in the presence of 2 mg/mL glucose in air.

and consequently H_2O_2, when no carbon source is present in the medium and no lactate is being produced? Further work is required to address this issue.

The Fe(III) produced by H_2O_2 was bound by *L. acidophilus* by a trypsin-sensitive binding site. This suggests some type of specificity, though a definitive statement on this issue must await further work. A surprising finding was the inability of *L. acidophilus* to bind substantial amounts of Fe(III) at 0 °C as opposed to 37 °C. It is well established that hydrophobic bonding weakens at lower temperatures, and our observations may indicate that hydrophobic bonding of a profoundly temperature-sensitive nature is involved in the interaction of a protein on the cell surface with Fe(III) in the form of $Fe(OH)_3$.

Our data support the hypothesis that, as bifidobacteria, *L. acidophilus* is capable of internalizing Fe^{2+} and oxidizing it intracellularly by putative ferroxidase(s). The transport mechanism is apparently trypsin-sensitive, while the ferroxidase(s) are not. The ferroxidase was, however, heat-sensitive. The binding of Fe(III) produced by the particulate fraction was apparently different from that produced by H_2O_2, since it was unaffected by trypsin. The particulate fraction, it should be noted, did retain its ability to bind H_2O_2-produced Fe(III), which was greatly diminished by trypsin digestion.

Hydrogen peroxide is antimicrobial largely through its ability to produce inorganic bacteriocidal substances such as hypochlorite and superoxide anion (Cords and Dychdala, 1993). Our experiments have shown that H_2O_2 is capable of an additional potentially antimicrobial function: withdrawal of the easily assimilable ferrous iron from the medium by converting it to the insoluble Fe(III). Such oxidation is also facilitated by lactate and by the intracellular ferroxidase(s). In the absence of oxygen, however, i.e., under anaerobic conditions such as those in the animal colon, lactobacilli and other probiotics could exert this effect only insofar as they are able to internalize Fe^{2+} without oxidizing it. Without such intracellular oxidation, the amount of Fe^{2+} internalized may be limited. An additional potentially antimicrobial action of *L. acidophilus* appears to be its ability to bind certain forms of Fe(III). Though some of this activity may be diminished by proteolytic digestion during passage through the small intestine, it was found that trypsin-treated cells were able to resume normal growth following reinoculation into the TPY medium. Thus, *L. acidophilus*, subjected to enzymatic action in the small intestine, should have no difficulty in reestablishing itself in the colon. Lactate, of course, may also withdraw iron from microorganisms; however, the extent to which it could be so effective with regard to pathogens is yet to be explored.

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