Binding of $Fe(OH)_3$ to *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus*: Apparent Role of Hydrogen Peroxide and Free Radicals

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Lactobacillus delbrueckii ssp. *bulgaricus* and *Lactobacillus acidophilus*, both used extensively as probiotics and to produce fermented milk products, were investigated for their ability to bind $Fe(OH)_3$ as a function of time in the presence and absence of approximately 200 μM H_2O_2 and in the presence and absence of glucose. There was a very rapid initial phase, followed by a second slow phase, and, when fermentable sugar was absent, a third rapid phase. Thermodynamic parameters were determined for the initial rapid binding phase using Scatchard plots. The binding process was entropy driven against an enthalpy barrier, indicating that hydrophobic-type bonding was involved. Results were similar for both species of lactobacilli, and in the presence or absence of glucose and/or H_2O_2. In the later phases, binding of $Fe(OH)_3$ was decreased if carried out without H_2O_2 in the medium. Free radicals, apparently produced from H_2O_2 and iron, were able to alter cell surfaces to allow for greater binding of $Fe(OH)_3$. The ability of probiotics to bind $Fe(OH)_3$ may serve to limit the availability of iron to pathogenic microorganisms.

Keywords: *Iron; $Fe(OH)_3$; lactobacilli; hydrogen peroxide; free radicals; probiotics*

INTRODUCTION

Homofermentative lactobacilli and bifidobacteria have been used in food industry to prepare fermented milk products and have been consumed by man and animals as probiotics for many years. In the latter capacity, these organisms are said to alleviate or prevent many acute and chronic diseases in humans, such as colon cancer, hypercholesterolemia, diarrhea, and liver disease among others (e.g., Mital and Garg, 1995; Tamime et al., 1995). In addition, probiotics exert inhibitory effects upon other microorganisms, especially intestinal pathogens and organisms involved in food spoilage (Saavedra, 1995; Giese, 1994; Hughes and Hoover, 1991; Daeschel, 1989). There is considerable interest in establishing the mechanisms by which probiotics may exert their beneficial effects *in vivo* and under food storage conditions.

Several mechanisms have been identified that explain the antimicrobial action of probiotics. This includes potentiation of the host immune response (Perdigon et al., 1995), but most involve the elaboration of various substances into the medium, which inhibit the growth of other microorganisms. The latter include organic acids, such as lactic acid, which lowers the pH of medium; bacteriocins; hydrogen peroxide, which may damage other organisms via free radical formation; and others (Wood, 1992; Daeschel, 1989). Some years ago, we proposed that the antibacterial action of bifidobacteria may also be associated with a withdrawal of iron from the medium, thus making it unavailable to pathogens (Bezkorovainy and Solberg, 1989). This concept was extended to hydrogen peroxide-producing lactobacilli, such as *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*). It was found that both organisms were able to oxidize ferrous iron to the insoluble and less available ferric form via an elaboration of hydrogen peroxide and that the resulting $Fe(OH)_3$ could become firmly bound to the bacteria (Kot et al., 1995, 1996).

Binding of ferric hydroxide by both organisms was concentration-dependent, and binding could be largely abolished by treating these cells with trypsin (Kot et al., 1995, 1996). In addition, binding was temperature-dependent. These findings suggested that these two species of lactobacilli had specific ferric hydroxide binding areas on their surfaces and suggested the possibility that the binding could be further investigated using classical physical-chemical approaches. Attempts were made to express the binding of $Fe(OH)_3$ by the lactobacilli via Scatchard plots (Scatchard, 1949); however, this was unsuccessful: our data indicated that under the conditions used, which involved metabolizing cells, ferric hydroxide binding continued over a long period of time without reaching an "equilibrium" (Kot et al., 1996). After a number of approaches were tried, we identified conditions under which the binding of $Fe(OH)_3$ by *L. bulgaricus* and *L. acidophilus* could be analyzed by Scatchard plots; it involves the characterization of the rapid initial binding phase only in short-term experiments in the presence and absence of a carbon source and in the presence or absence of hydrogen peroxide. The results of these investigations constitute the substance of the present report.

MATERIALS AND METHODS

Microorganisms. *L. bulgaricus* (ATCC 11842) and *L. acidophilus* (ATCC 4386) were purchased from American Type Culture Collection, Inc. (Rockville, MD). These organisms were grown in the trypticase−phytone−yeast extract (TPY) medium described by Scardovi (1986). Glucose was used routinely as a carbon source at the level of 2 mg/mL. Identity of *L. bulgaricus* was periodically confirmed by Gram-staining and microscopic examination, fermentation of lactose in addition to glucose, production of D-(−)-lactate rather than L-(+)-lactate, and production of H_2O_2.

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S0021-8561(96)00605-X CCC: $14.00

The cells were grown in 120 mL bottles at 37 °C under anaerobic conditions for 18 h (post-logarithmic phase), when the turbidity of the cultures, measured at 610 nm, reached 1.2. The cells were then centrifuged down at 4 °C and washed once with ice-cold 0.9% NaCl solution. Following centrifugation, the cells were suspended in a 0.1 M 3,3-dimethylglutarate buffer solution at pH 6.5, which also contained 0.4 g of KCl, 8 g of NaCl, and 0.14 g of $CaCl_2$ per liter. The A_{610} of this suspension was 1.2.

Metabolically inactive cells were prepared by heating cell suspensions in the above buffer solution at 85 °C for 15 min. Such cells produced neither lactate nor H_2O_2.

Ferric Hydroxide Solutions. Ferric hydroxide solutions were prepared by oxidizing $^{59}Fe^{2+}$ with H_2O_2 as follows: to 75 mL of the pH 6.5 buffer solution was added 1.4 μL of 30% H_2O_2. After mixing, 2.25 mL of 10 mM $^{59}FeSO_4$ was added with constant mixing. A clear amber-colored solution ensued immediately. This provided a 291 μM $Fe(OH)_3$ stock solution containing an excess of 188 ± 137 μM H_2O_2 (n = 13) and containing no Fe^{2+} as revealed by the Ferrozine color reaction (Kot et al., 1996). This solution was freshly prepared before use. These $Fe(OH)_3$ solutions (more likely colloidal suspensions) were quite stable during the time span of the experiment: when incubated in the absence of cells but in the presence of kaolin or cellulose, iron was neither precipitated nor could be spun down with these insoluble substances. When it was desired to remove excess hydrogen peroxide from the $Fe(OH)_3$ solutions, they were treated with 233 units/mL (0.15 mg/mL) of mouse liver catalase. Alternately, $Fe(OH)_3$ solutions with undetectable H_2O_2 content were prepared by titrating 300 μM H_2O_2 in the 3,3-dimethylglutarate buffer solution with 10 mM $FeSO_4$ until no H_2O_2 could be detected. Such solutions contained no detectable ferrous iron either.

Ferric Hydroxide Binding Assays. Cell suspensions in the pH 6.5 3,3-dimethylglutarate buffer solution with or without various additives were preincubated at 37 °C for 30 min to metabolize any endogenous glucose, centrifuged, resuspended in half the original volume of the pH 6.5 buffer solution, and combined with an equal volume of prewarmed or precooled $^{59}Fe(OH)_3$ solution appropriately diluted to give the desired final iron concentration. Final cell suspensions had an A_{610} of 1.2 and represented 4.5 ± 0.21 mg dry bacterial weight per 5 mL of suspension. If required, solid glucose or another sugar was added at the same time to a final concentration of 2 mg/mL. Final H_2O_2 concentrations did not exceed 200 μM, unless the iron solution had been pretreated with catalase. 5 mL samples were then withdrawn at various time intervals and cooled on ice, and the cells were centrifuged down at 4 °C, washed with ice-cold pH 6.5 buffer solution, and counted in a Gamma 4000 counter (Beckman Instruments, Palo Alto, CA). Occasionally, the $Fe(OH)_3$ binding experiments were done under nitrogen atmosphere as previously described (Kot et al., 1994).

Calculations of Thermodynamic Parameters. For the purpose of calculating thermodynamic parameters, the binding of $Fe(OH)_3$ by *L. bulgaricus* and *L. acidophilus* was carried out at 4, 27, 37, and 47 °C at various iron concentrations but at a constant cell density as described above.

Binding of $Fe(OH)_3$ by the lactobacilli was investigated for the initial rapid binding phase only. For this purpose, samples were collected for each $Fe(OH)_3$ concentration for the first 30 min only, and straight lines were fitted using the least-squares method with a programmed minicomputer (Hewlett-Packard model HP 32SII). From the straight-line equation displayed, the y-intercept was determined representing the quantity of $Fe(OH)_3$ bound instantaneously. This "bound" $Fe(OH)_3$ was then subtracted from total $Fe(OH)_3$ present in the medium to obtain "unbound" (free) $Fe(OH)_3$.

The bound and unbound $Fe(OH)_3$ values (normally 5) at each temperature were then handled by the Scatchard procedure (Scatchard, 1949) to provide the association constants (K_a's) and the number of binding sites. The latter were expressed in nmol/mg bacterial dry weight. Gibbs free energy changes at each temperature could be calculated by the well-known equation:

$$\Delta G_o' = -RT \ln K_a$$

and entropy ($\Delta S_o'$) and enthalpy ($\Delta H_o'$) changes were calculated graphically using

$$\Delta G_o' = \Delta H_o' - T\Delta S_o'$$

Materials and Analytical Procedures. Trypticase and peptone, used for growth medium preparation, were obtained from BBL Laboratories (Cockneysville, MD), and $^{59}Fe^{2+}$ came from DuPont Laboratories (Boston, MA).

The source of *Ginkgo biloba* was a local pharmacy. The capsules are distributed by NOW Foods Co. (Glendale Heights, IL). Three capsules (180 mg) were extracted with a mixture of 19 mL of the pH 6.5 buffer solution plus 1 mL of ethanol for 24 h and then filtered to obtain a clear intensely yellow solution (extract). 1 mL of this extract (9 mg of *G. biloba* material) was routinely used for each 30 mL of cell suspension. *G. biloba* is a food supplement containing a group of flavonoid-type compounds (Cook and Samman, 1996). This material has been used as an antioxidant and free radical scavenger (Bors et al., 1996).

Catalase was of mouse liver origin and contained 1600 units of activity/mg of protein, 22 mg of protein/mL. Superoxide dismutase was of horseradish origin containing 3000 units/mg of protein. Both were purchased from Sigma Corp. (St. Louis, MO). All other chemicals and reagents were also purchased from Sigma Corp.

Ferrous iron was measured with Ferrozine using Sigma kit No. 690-A. D-(−)-Lactate was measured using D-lactate dehydrogenase, and H_2O_2 was quantitated using the Sigma cholesterol kit No. 352, all as previously described (Kot et al., 1996). The OH free radical was detected colorimetrically using hydroxylation of salicylate to 2,3-dihydroxybenzoate as a criterion (Richmond et al., 1981).

RESULTS

Hydrogen Peroxide Production and Sensitivity by *L. bulgaricus*. In our previous report it was indicated that 5 mL of a *L. bulgaricus* suspension with an A_{610} of 1.2 produced 288 ± 90.5 μM H_2O_2 after incubation for 1 h in the presence of 2 mg/mL glucose (Kot et al., 1996). Viability of the organisms was not affected by this H_2O_2 concentration, and peroxide concentrations in this range may be considered to be normal in the *L. bulgaricus* environment. A study of lactic acid production as a function of initial H_2O_2 concentration in the medium indicated that the organism could tolerate up to 500 μM H_2O_2, beyond which lactate production showed a decline. Half-maximal lactate production was observed at an H_2O_2 concentration of about 740 μM. In subsequent $Fe(OH)_3$ binding experiments, the H_2O_2 concentration was 201 ± 116 μM, unless the iron solutions had been pretreated with catalase or prepared without detectable H_2O_2.

It is interesting to note that H_2O_2 concentrations in such binding experiments showed a tendency to decline as a function of time; the lower the initial H_2O_2 concentration, the faster the the rate of observed loss of the peroxide from the medium. In one such experiment, where cells were incubated with 120 μM $Fe(OH)_3$ and 190 μM H_2O_2, the peroxide concentrations were 150, 110, 80, and 40 μM after 30, 60, 120, and 150 min of incubation, respectively. There was no disappearance of hydrogen peroxide in the absence of $Fe(OH)_3$ or from $Fe(OH)_3$ solutions without cells.

Binding of $Fe(OH)_3$ to *L. bulgaricus* as a Function of Time. Figure 1 illustrates the binding of exogenously-produced $Fe(OH)_3$ by *L. bulgaricus* as a function of time in the presence of H_2O_2 and varying concentrations of $Fe(OH)_3$, but in the absence of glucose.

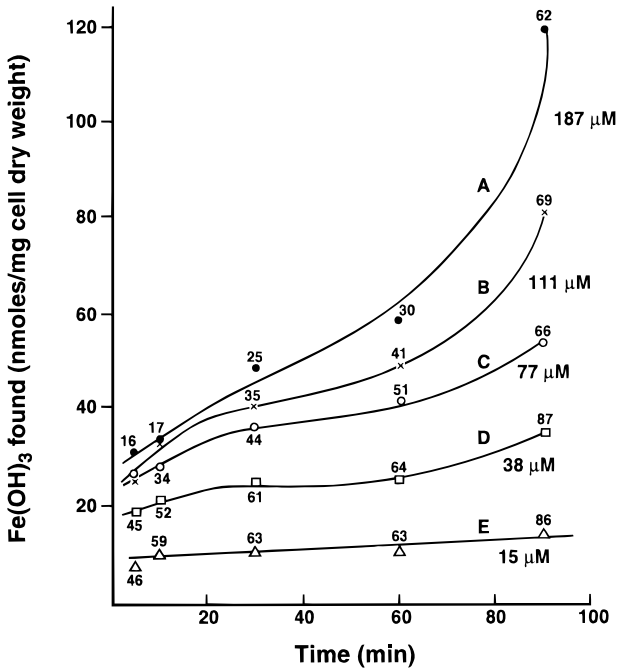

Figure 1. Iron hydroxide binding to *L. bulgaricus* as a function of time and iron concentration in the absence of fermentable sugar. Initial $Fe(OH)_3$ concentrations are indicated with each curve in μM. The smaller numbers corresponding to specific points on the curves indicate the percent of initial iron that was bound by the bacteria. Incubations were carried out at 37 °C in air at pH 6.5. Bacterial densities were 4.5 mg dry weight per 5 mL suspension ($A_{610} = 1.2$).

The binding curves, especially at higher $Fe(OH)_3$ concentrations, were triphasic: there was a rapid initial binding phase, after which the binding increased gradually with time. After about 60−90 min, a sharp increase in binding was observed. At lower $Fe(OH)_3$ concentrations, this S-shaped curvature was less evident. Similar results were observed with *L. acidophilus* (data not shown).

Another phenomenon obvious from Figure 1 was the increased proportion of $Fe(OH)_3$ bound to that present in the medium as $[Fe(OH)_3]$ originally in the medium decreased. With higher iron concentrations, the percentage of bound iron declined, though the absolute amounts bound were increased, as expected. Thus, at $[Fe(OH)_3] = 15-38$ μM, some 86−87% of the iron in the medium could be mopped up after 90 min. After only 10 min, some 59% was bound when the iron concentration was 15 μM. Yet only 17% became bound when iron concentration was 181 μM.

Experiments depicted in Figure 1 were performed in the absence of glucose, yet the cells could be metabolically reactivated after 60 or 90 min, and occasionally after 120 min incubations, by the addition of 2 mg/mL glucose. Rates of lactic acid production under such circumstances were equivalent to those of controls that initially contained glucose (data not shown).

Figure 2 illustrates the binding of $Fe(OH)_3$ by *L. bulgaricus* in the presence of fermentable sugars glucose and lactose. For comparison purposes, curves representing iron binding in the absence of any sugar and with galactose, a nonfermentable sugar, are also shown. The curve representing the sugar-free system is reminiscent of curves seen in Figure 1, while the curves obtained in the presence of glucose and lactose show a relatively low level of iron binding. The curve representing iron binding in the presence of galactose showed

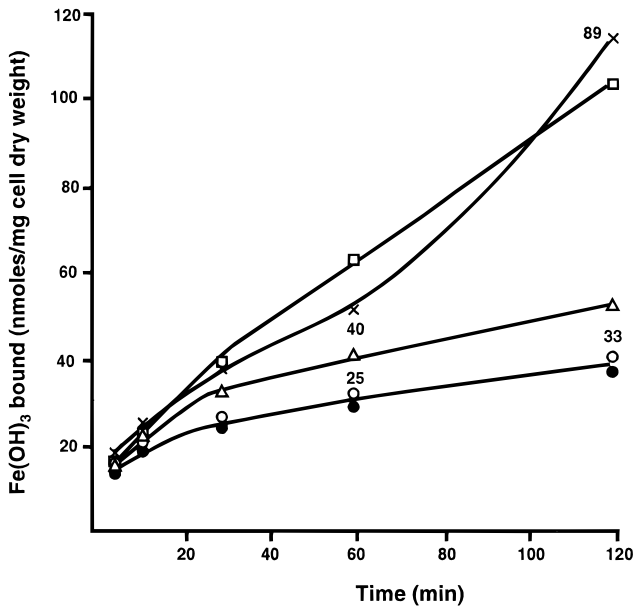

Figure 2. Binding of $Fe(OH)_3$ by *L. bulgaricus* as a function of time in the presence and absence of fermentable sugars. Initial iron concentrations were 114−119 μM. Incubations were carried out at 37 °C in air at pH 6.5. Small numbers opposite some points on the curves indicate the percent of initial iron that was bound by the bacteria. (○) Binding in the presence of 2 mg/mL glucose; (●) binding in the presence of 2 mg/mL lactose; (□) binding in the presence of 2 mg/mL galactose; (△) binding in the presence of 2 mg/mL mannose; (×) binding in the absence of any sugar.

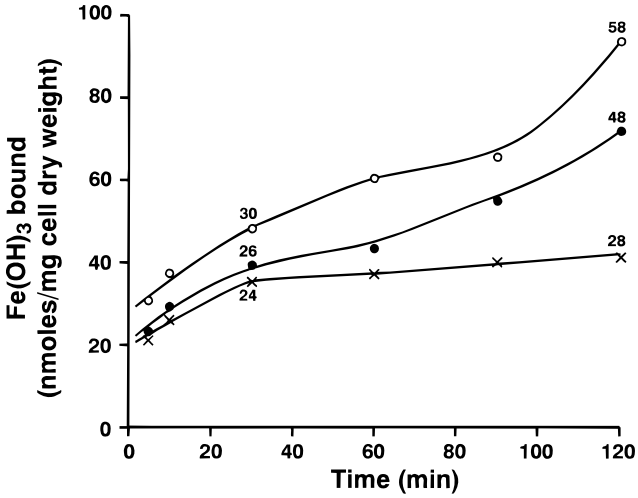

Figure 3. Binding of $Fe(OH)_3$ by *L. bulgaricus* as a function of time in the presence and absence of H_2O_2 and glucose. Incubations were carried out at 37 °C in air at pH 6.5. Small numbers indicate the percent of initial iron that was bound by the bacteria. (○) Binding in the presence of H_2O_2, with no glucose, [Fe] = 150 μM; (●) binding in the presence of undetectable amounts of H_2O_2, no glucose, [Fe] = 138 μM; (×) binding in the presence of undetectable amounts of H_2O_2, in the presence of 2 mM glucose, [Fe] = 139 μM.

approximately the same quantity of $Fe(OH)_3$ bound as that of the sugar-free system; its shape, however, was not necessarily the same. There was an insufficient number of experimental points to tell whether or not the two curves were truly different. Nevertheless, the rapid phases of $Fe(OH)_3$ binding were at very similar levels in all cases.

Figure 3 illustrates the binding of $Fe(OH)_3$ by *L. bulgaricus* cells in the absence of detectable H_2O_2. For comparison purposes, a curve representing $Fe(OH)_3$

Table 1. Thermodynamic Constants for the Binding of $Fe(OH)_3$ by *L. bulgaricus*

temperature (°C)	maximum no. of binding sites (nmol/mg dry weight)	$K_a \times 10^4$ (M^{-1})	$\Delta G_o'$ (cal/mol)	$\Delta S_o'$ (eu)	$\Delta H_o'$ (cal/mol)
		With H_2O_2 and Glucose (2 mg/mL)			
27[a]	19.5 ± 4.81[b]	0.970 ± 0.442	−5420 ± 277	43.1[c]	+7400[c]
37	23.3 ± 7.53	2.81 ± 2.20	−6150 ± 507		
47	59.8 ± 16.5	2.81 ± 2.65	−6280 ± 594		
		With H_2O_2, Without Glucose			
4	15.2 ± 7.30	0.416 ± 0.305	−4490 ± 151	46.4[d]	+8350[d]
27	22.1 ± 8.10	1.23 ± 0.550	−5560 ± 281		
37	39.9 ± 15.3	2.68 ± 1.13	−6190 ± 388		
47	59.2 ± 20.1	2.65 ± 1.27	−6410 ± 342		
		Without Detectable H_2O_2 and Glucose			
4	8.91 ± 3.46	0.900 ± 0.603	−4910 ± 361	40.9[e]	+6470[e]
27	31.1 ± 9.57	1.92 ± 0.960	−5830 ± 275		
37	44.8 ± 9.02	1.93 ± 1.21	−5980 ± 375		
47	49.1 ± 16.3	5.095 ± 3.23	−6790 ± 414		

[a] Scatchard plots at 4 °C were irreproducible. [b] Standard deviations. [c] Entropy and enthalpy changes assumed to be temperature independent. Calculated from $\Delta G_o' = \Delta H_o' - T\delta S_o'$ plot, correlation of curve = 0.93. [d] Same assumptions and calculations as in footnote *c*. Correlation coefficient of curve = 0.99. [e] Same assumptions and calculations as in footnote *c*. Correlation coefficient of curve = 0.98.

Table 2. Effect of Catalase and Various Free Radical Antagonists on the Binding of $Fe(OH)_3$ by *L. bulgaricus*[a]

time of incubation (min)	additions						
	catalase[b]	under N_2[c]	under N_2 with catalase[d]	superoxide dismutase[e]	cytochrome *c*[f]	mannitol[g]	*G. biloba* extract[h]
5	137 ± 30.4	−	205	262	121 ± 15.2	91	101 ± 27.6
10	118 ± 25.3	99	141	287	155 ± 46.0	91	116 ± 19.6
30	102 ± 19.5	116	152	250	125 ± 37.3	94	77.5 ± 21.9
60	91.7 ± 18.6	98	107	176	109 ± 23.9	81	63.5 ± 12.6
90	89.0 ± 11.5	100	91	126	70.0 ± 1.00	82	52.0
120	77.6 ± 5.13	97	89	116	79.4 ± 13.9	87	48.8 ± 10.4

[a] All experiments were carried out in air at 37 °C. The results are expressed as percent of $Fe(OH)_3$ bound by the lactobacillus in the absence of glucose, which thus served as a control. Standard deviations are provided wherever possible. [b] The $Fe(OH)_3$ solution was treated with catalase before it was incubated with the cells. Catalase concentration was 233 units (0.15 mg) per mL. $Fe(OH)_3$ concentration in the catalase-treated samples was 132 ± 15.7 μM, while that of the control was 134 ± 8.77 μM. [c] Average of two experiments. $Fe(OH)_3$ concentration for the sample and control was 111 μM. [d] Catalase concentration same as in footnote *b*. Average of two experiments. $Fe(OH)_3$ concentration was 135 μM for the samples and 144 μM for the controls. [e] Average of two experiments. Concentration of superoxide dismutase was 3 mg/mL, that of $Fe(OH)_3$ for the samples was 137 μM, and that for the control was 134 μM. [f] Concentration of cytochrome *C* was 4.0 mg/mL, that of $Fe(OH)_3$ for the samples was 120 ± 15.9 μM, and that for the controls was 115 ± 9.20 μM. [g] Average of three experiments. Concentration of mannitol was 2 mg/mL, that of $Fe(OH)_3$ for the samples was 133 μM, and that of controls was 134 μM. [h] Concentration of *G. biloba* was 0.3 mg per mL of incubation medium, that of $Fe(OH)_3$ for the samples was 130 ± 1.87 μM, and that of the controls was 113 ± 8.80 μM.

binding in the presence of approximately 200 μM H_2O_2 is also shown. It will be seen that in all cases where H_2O_2 was not detectable, iron binding was less extensive than in otherwise comparable situations where H_2O_2 was present. This lower level of iron binding was not as evident after short periods of incubations as it was after 30−120 min.

Binding Constants and Thermodynamic Parameters. Table 1 illustrates the calculated constants for $Fe(OH)_3$ binding by *L. bulgaricus*. Values for *L. acidophilus* were very similar (data not shown). These apply to the initial rapid phase only. It may be seen that the binding was entropy driven against an enthalpy barrier. The number of binding sites increased as temperature was increased, and the same pattern was followed by the association constants. Qualitatively, little if any difference is obvious among respective binding parameters obtained in the presence or absence of H_2O_2 and glucose.

Apparent Effects of H_2O_2 and Free Radicals on the Binding of $Fe(OH)_3$ by Lactobacilli. Hydrogen peroxide is toxic to many organisms, though *L. bulgaricus* and *L. acidophilus*, themselves H_2O_2 producers, can withstand considerable concentrations thereof as indicated above. This does not mean, however, that H_2O_2 could not in some way affect bacterial cell surfaces and therefore $Fe(OH)_3$ binding. Thus, Figure 3 illustrates that cells in H_2O_2-containing medium bound more $Fe(OH)_3$ than in media containing little if any H_2O_2 after prolonged incubation times. Additionally, H_2O_2 was removed from the $Fe(OH)_3$ solutions with catalase before they were used with bacterial cells for binding studies in the absence of glucose. At $Fe(OH)_3$ concentrations of about 50 μM and higher, lack of H_2O_2 resulted in an enhancement of the initial rapid binding phase and a substantial abolition of the terminal rapid binding phase. Thus, even though such cells initially bound more $Fe(OH)_3$ than did cells in the presence of H_2O_2, the total amount of $Fe(OH)_3$ bound after 60−120 min of incubation was smaller (Table 2). At lower $Fe(OH)_3$ concentrations, the extent of its binding by the H_2O_2-free suspensions was in fact lower than that in the presence of peroxide (data not shown). The data obtained using catalase-treated $Fe(OH)_3$ solutions did not lend themselves to an analysis via the Scatchard plot.

The action of H_2O_2 is often associated with the production of free radicals, especially in the presence of iron (Floyd, 1993). It was therefore possible that the effects observed with H_2O_2 on the bacterial cells were really due to free radical action. Hydroxyl free radicals were detected by the method of Richmond et al. (1981) when ferrous iron was oxidized by excess H_2O_2, but not when the $Fe(OH)_3$ solutions were prepared to leave no

detectable H_2O_2. Several potential free radical scavengers, such as cytochrome *C*, mannitol, superoxide dismutase, and the *G. biloba* extract (Bors et al., 1996; Ciba Foundation, 1978) were used in $Fe(OH)_3$ binding studies. Their effects, to a greater or less extent, paralleled those of catalase at relatively high $Fe(OH)_3$ concentrations, if data presented in Table 2 are compared. This was especially true of superoxide dismutase in the initial phase of binding, and of *G. biloba* extract in the later stages. The results obtained with the latter were almost identical to those seen with glucose (see Figure 2). Mannitol had little if any influence. Ferric hydroxide binding studies were also done in an oxygen-poor atmosphere. Nothing unusual was observed. Exclusion of H_2O_2 via catalase treatment of the iron solution resulted in binding of $Fe(OH)_3$ that differed but little from that observed in air. The above results are also summarized in Table 2.

Free radical scavengers used herein had no toxic effects on *L. bulgaricus*. Following 60 or 90 min incubations with $Fe(OH)_3$ in the presence of these compounds or of catalase, but in the absence of glucose, the adition of the latter resulted in a resumption of lactic acid production at normal rates (data not shown).

Effect of Various Carbohydrates on the Binding of $Fe(OH)_3$ by *L. bulgaricus*. Figure 2 illustrates the effect of various sugars on the binding of $Fe(OH)_3$ by *L. bulgaricus*. Glucose and lactose are normally fermented by this organism, while galactose and mannose are not supposed to be (Kandler and Weiss, 1986). Relative to the sugar-free system, glucose and lactose had a profoundly negative effect on iron binding, especially beyond the initial rapid phase. Galactose behaved as the sugar-free system, while mannose was in between. Both glucose and lactose did produce lactate, galactose produced no lactate, and mannose produced some, amounting to 14−20% that of glucose over a 1 h incubation period.

At least a part of the fermentable sugar effect may be attributable to lactic acid production by the bacteria, since lactic acid was shown to inhibit $Fe(OH)_3$ binding: initially, in the presence of 750 μg/mL of lactic acid at pH 6.5 (maximum that was produced by *L. bulgaricus* after 120 min of incubation), the binding of $Fe(OH)_3$ (at the initial level of 140 μM) was only 54% that of control, and this inhibitory effect of lactic acid was concentration dependent. Glucose itself had no effect on the concentration of H_2O_2, i.e., incubation of 200−400 μM H_2O_2 with 2 mg/mL glucose for up to 120 min did not result in any significant decline in H_2O_2 concentration at pH 6.5.

Interestingly, if glucose alone or glucose with a free radical scavenger such as the *G. biloba* extract was added to an $Fe(OH)_3$ binding experiment begun 60 min earlier without any fermentable sugar, there was no change in the progress of the binding process: even though lactic acid was being produced normally, the system behaved as if no glucose had been present (data not shown).

Removal of Iron from Iron-Loaded *L. bulgaricus*. The binding of $Fe(OH)_3$ by lactobacilli may be fortuitous, or it may have physiological purposes. Winkelmann (1979) studied ferric hydroxide polymer binding to certain fungal strains and found that their metabolic end products, hydroxy acids, were able to mobilize Fe(III) from the surface of the cells and thereby make the iron available to the organism for growth purposes. To investigate whether or not $Fe(OH)_3$ bound to lactobacilli could also be mobilized by their metabolic end product, lactic acid, *L. bulgaricus* was loaded with $Fe(OH)_3$ and the cells were then incubated with lactic acid as well as other metal chelators. The results of these studies are depicted in Table 3. It is seen that lactate mobilized a small amount of iron; however, the effects of citrate and ethylenediaminetetraacetic acid (EDTA) were much more impressive. Neither EDTA nor citrate is a product of lactic acid bacterial metabolism. The *G. biloba* extract did not remove any iron from the cells under these conditions, even though flavonoids are supposed to be iron chelators (Bors et al., 1996).

Table 3. Elution of Iron from $Fe(OH)_3$-Preloaded *L. bulgaricus* by Various Chelating Agents (in Percent of Initial Cellular Iron Content)[a]

agent	concentration (mM)	time of elution (min) 30	60	90
citrate	2	45	59	63
EDTA[b]	2	55	79	79
lactate[c]	2	−	−	47
	4	10	−	−
	8.3	13	−	−
	11	20	−	−
	83	70	−	−
pH 5.0 buffer[d]	−	−	−	19
pH 6.5 buffer[d]	−	5	−	5

[a] Cells were preincubated with 110−120 μM $Fe(OH)_3$ for 60 min at 37 °C in the absence of glucose. There was 40−50 nmol of iron bound per mg dry cell weight. The cells were washed with ice-cold pH 6.5 buffer solution and then incubated with the chelators at pH 6.5 and 37 °C. [b] Ethylenediaminetetraacetic acid. [c] Under normal conditions, the amount of lactate produced was 8.3 mM after 2 h of incubation at 37 °C in the presence of 2 mg/mL glucose. [d] 0.1 M 3,3-dimethylglutarate.

Table 4. Binding of $Fe(OH)_3$ by Heated (Metabolically Inactive) *L. bulgaricus* Cells as a Function of Time in the Absence of Glucose[a]

cell type	[Fe] (μM)	time (min) 5	10	30	60	90	120
In the Presence of H_2O_2							
heated	100	46.4	53.3	62.4	76.0	−	110
control	112	38.0	44.9	51.8	63.3	86.2	111
With No Measurable H_2O_2							
heated	119	39.3	47.1	55.6	60.2	65.6	86.2
control	113	30.1	36.4	41.1	52.0	66.2	85.1

[a] Heating at 85 °C for 15 min. In nmol of $Fe(OH)_3$ bound per mg dry cell weight.

Ferric Hydroxide Binding by Metabolically Inactive (Heated) *L. bulgaricus*. Cells were heated in the pH 6.5 dimethylglutarate buffer solution at 85 °C for 15 min and then subjected to $Fe(OH)_3$ binding as a function of time. The results are depicted in Table 4. Both in the presence and apparent absence of H_2O_2, ferric hydroxide binding by heated cells was higher than that of controls, though toward the end of the experiment (90−120 min), the binding level became almost identical. Also, binding in the presence of H_2O_2, both for heated and unheated cells, was higher than in its apparent absence. Heated cells were not able to produce lactic acid or H_2O_2. It is noteworthy that ferrous iron uptake by lactobacilli (and bifidobacteria) is abolished following heating of cells (Kot et al., 1995); binding of $Fe(OH)_3$ by heated cells, on the other hand, is augmented following heating.

DISCUSSION

Both *L. bulgaricus* and *L. acidophilus* were able to bind $Fe(OH)_3$; however, the quantity bound and mode

of binding depended heavily on conditions used. In the presence of a metabolizable carbon source, the binding of $Fe(OH)_3$ was relatively small as shown in this work and previously (Kot et al., 1996). The observed effect of fermentable sugars may have been, at least in part, due to the presence of lactate, which sequesters iron and makes it unavailable for binding. Additionally, the elaboration of a "stress" protein (Condon, 1987) by the microorganisms may have protected them against free radical alteration of their surfaces. Nevertheless, the level of the initial rapid phase of iron binding was the same for lactobacilli in the presence and absence of glucose, and it was in the later phases of binding that the differences were most pronounced.

The binding of small ligands by larger substances has traditionally been studied by the method of equilibrium dialysis using semipermeable membranes. When an equilibrium is established, concentrations of the small molecular-weight substance is measured on both sides of the membrane and the data are then handled by the Scatchard method (Scatchard, 1949). Scatchard-type plots have also been used to study macromolecule-cell interactions, e.g., binding of transferrins to reticulocytes (Kornfeld, 1969), or to the K562 cells (Klausner et al., 1983). No semipermeable membranes were used in these and numerous other similar studies; one merely incubated the cells with the labeled ligand, then separated the cells from the medium, and determined the amount of ligand bound. In this study too, the macromolecular $Fe(OH)_3$, which has a molecular weight near 150 000 (Spiro et al., 1967), was permitted to interact with bacterial cells, the cells were then separated from the medium, and the bound $Fe(OH)_3$ was determined. The results for the initial rapid binding phase were amenable to analysis by the Scatchard plot, permitting us to calculate the thermodynamic constants for the binding reaction.

Results of these studies indicated that $Fe(OH)_3$ binding by the cells, both in the presence and absence of H_2O_2 and glucose, was entropy driven (Table 1), suggesting a randomization of water molecules and a hydrophobic association of $Fe(OH)_3$ with the cell surface. That bonding is of the hydrophobic type is also indicated by the fact that the free energy of binding decreases (becomes more negative) as the temperature is raised. Hydrophobic bonds become stronger with increasing temperatures. Scatchard plots also indicated a finite number of iron binding sites, whose number increased with temperature, but whose exact nature is unknown at the present. However, trypsin digestion results (Kot et al., 1995, 1996) have suggested that peptidic material associated with cell walls or membranes may be involved in the binding process. Though all values reported in Table 1 have high standard deviations, the temperature-related trends are unambiguous.

The progress of $Fe(OH)_3$ binding by lactobacilli beyond the initial rapid phase was biphasic in the absence of fermentable sugar. Beyond the initial rapid binding phase, there was apparently a slow opening of binding sites, followed by a rapid opening after 60−90 min of incubation (see Figure 1). This was observed even in the presence of undetectable amounts of H_2O_2 (Figure 3). The third rapid binding phase, observed in the absence of fermentable sugar, was most likely caused by cell death, since both heated and unheated cells were able to bind identical amounts of $Fe(OH)_3$ after 90−120 min (Table 4).

The binding of $Fe(OH)_3$ by lactobacilli was enhanced by H_2O_2, whose action is normally associated with the production of free radicals. Though H_2O_2 may be considered a normal component of bacterial environment, it was, nevertheless, able to alter the bacterial surface to allow higher levels of $Fe(OH)_3$ binding. This is obvious from the results shown in Figure 3 and Table 4, where $Fe(OH)_3$ solutions free of detectable H_2O_2 were used, and from free radical scavenger experiments described in Table 2. The results observed with free radical scavengers and solutions free of detectable H_2O_2 were somewhat different: while the latter were amenable to Scatchard-type analysis, the former were not, and, in addition, free radical scavenger and catalase actions showed very high $Fe(OH)_3$ binding levels in the initial phase of the binding curve (Table 2). These discrepancies have not been resolved; it may be possible that $Fe(OH)_3$ solutions prepared with undetectable final H_2O_2 concentrations and used in experiments depicted in Figure 3 were still able to generate free radicals albeit at lower levels, or, on the other hand, the actions of such enzymes as catalase and superoxide dismutase may include effects other than just the dismutation of H_2O_2 and/or the superoxide anion. In the end, however, with one exception, less $Fe(OH)_3$ is bound by lactobacilli in the absence of free radical action than in its presence (see Table 2).

This work has shown that lactate produced by the lactobacilli can both significantly diminish the binding of $Fe(OH)_3$ by the cells and to remove some Fe(III) already bound to the cell surface. Whether or not lactate-bound iron can be used to supply iron to lactobacilli for growth purposes as citrate or malate can in fungi (Winkelmann, 1979) remains to be determined, if indeed iron is required for their growth and/or metabolism. It has been established that significant amounts of lactate-bound Fe(III) are not internalized or bound by *L. bulgaricus* or by *L. acidophilus* (Kot et al., 1995, 1996), though the uptake of smaller amounts of lactate-bound Fe(III) cannot be excluded. Regardless of whether the binding of $Fe(OH)_3$ by probiotics such as the lactobacilli is fortuitous or has evolved to serve a physiological purpose, the fact remains that $Fe(OH)_3$ is removed from the environment and may be withheld from harmful microorganisms. This effect is maximized in air, where the production of H_2O_2 and free radicals is possible. However, even in the absence of H_2O_2 such as would be the case under anaerobic conditions, the binding of $Fe(OH)_3$ by probiotics is still substantial via the initial rapid phase, whose magnitude is at least the same as that in the presence of free radicals, as shown in Figure 3 and Table 2. It should also be noted that a large proportion of ingested probiotics becomes inactivated during passage through the gastrointestinal tract, and that at least in one case, only about 25% survived to become established in the colon (Pochart et al., 1992). The inactivated lactobacilli, as per Table 4, are capable of binding larger amounts of $Fe(OH)_3$ than live cells, and this provides an additional capability to sequester iron by these organisms. Probiotics such as *L. acidophilus* and bifidobacteria are also capable to internalizing rather large amounts of ferrous iron (Kot et al., 1994, 1995), and this, along with their ability to bind $Fe(OH)_3$, may represent an effective iron-withholding host-defensive system against pathogen proliferation.

ACKNOWLEDGMENT

The authors are grateful to Mr. Edwin A. Metzl for technical assistance and helpful discussions.

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Received for review August 12, 1996. Accepted December 20, 1996.[⊗]

JF9606058

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.