Binding of Ferric Iron to the Cell Walls and Membranes of *Bifidobacterium thermophilum*: Effect of Free Radicals

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Bifidobacterium thermophilum (ATCC 25866) was incubated with 100−120 μM $^{59}FeSO_4$ and 300 μM excess of H_2O_2 for up to 120 min in the presence and absence of glucose. Samples were withdrawn after 5, 30, 60, and 120 min. These were protoplasted and ^{59}Fe(III) measured in the supernatant fraction (cell walls) and protoplasts (cell membranes). These experiments were also repeated in the presence of 400 μM Al(III), which, in whole cells, caused an increase in Fe(III) binding. The amount of iron in the cell wall fraction was constant regardless of time of incubation, was unaffected by Al(III), and was reduced by ~20% by glucose. On the other hand, the amount of iron on the protoplasts increased with time and was affected by both Al(III) (upward) and glucose (downward). Scatchard plots indicated that the number of Fe(III) binding sites on the cell walls was 37.6 nmol/mg of dry cell weight at zero time, whereas that of cell membranes was $^1/_{10}$ of that. It was concluded that Fe(III) binding by bifidobacterial cell walls was instantaneous and marginally dependent on free radical action. That of the cell membranes was time-dependent and was due to lipid peroxidation initiated by free radicals. Bifidobacteria can therefore function in the intestinal tract as probiotics by making $Fe(OH)_3$ unavailable to pathogens and by moderating free radical activity in the intestinal tract.

Keywords: *Iron; probiotics; Bifidobacterium thermophilum; free radicals*

INTRODUCTION

Bifidobacteria and lactobacilli are Gram-positive anaerobes that are normally present in the gastrointestinal tracts of humans and animals. They are considered to be beneficial to the health of their hosts and have been referred to as "probiotics" (Fuller, 1991). Probiotics of animal origin have been used extensively for the improvement of food production by farm animals (Jin et al., 1997) and, especially, as replacements for antibiotics in the maintenance of animal health (Fuller, 1999). In humans, probiotics have been used to treat certain types of diarrhea (Saavedra, 1995) and have been incorporated into various types of "functional foods" (Sanders, 1998). In the latter context, the low survivability of probiotic organisms in such functional foods as yogurts has been of considerable concern (Dave and Shah, 1997; Micanel et al., 1997) and has been attributed in part to the effects of oxygen-derived toxic products (Shah, 1997; Kailasapathy and Rybka, 1997). There is also a great deal of interest on whether probiotic therapy will affect the bioavailability of metals in animals, especially calcium and iron. In the latter case, such effects are unclear and may depend on specific strains of probiotics used (Oda et al., 1994). Research on probiotics and their effects on human and animal health are thus of considerable interest to agricultural, food, and nutritional science communities.

Bifidobacterium thermophilum has been isolated from bovine rumen contents and has been used as a probiotic in farm animals (Fuller, 1999). The basis for its probiotic action, as well as that of other bifidobacterial species, is not well understood. Our laboratory has suggested that the sequestration of iron by these bacteria may be the basis of one such mechanism (Bezkorovainy and Solberg, 1989). This may be so because partial removal of iron from the intestinal contents may make it less available to pathogens, the virulence of which depends on iron, and, in addition, sequestration of iron may abate the noxious effects of oxygen-based free radicals. Production of the extremely toxic OH free radical depends on the availability of Fe(II) and/or Fe(III) (Minotti and Aust, 1988). The sequestration of iron by bifidobacteria and the role of free radicals in this process have thus been of considerable interest to our laboratory.

There is mounting evidence to indicate that the binding of Fe(III) to the surface of *B. thermophilum* and, by extension, to other bifidobacterial species, is, at least in part, mediated by the action of free radicals (Kot and Bezkorovainy, 1998; Bezkorovainy and Kot, 1998). Thus, the iron, with or without exogenous H_2O_2, acts as a producer of free radicals and then becomes bound to cell surfaces if it is present in the form of $Fe(OH)_3$. It is not known whether both the cell walls and membranes are involved in the free radical-mediated binding of iron. An understanding of this phenomenon could be of significance in the assessment of the value of bifidobacteria as probiotics under anaerobic conditions, such as those seen in the colon, and may provide certain clues as to their survivability in an oxygen atmosphere and/or in the presence of H_2O_2.

The current paper deals with the distribution of Fe(III) following incubation with *B. thermophilum* in the presence of glucose and/or Al(III). The latter is an enhancer of membrane lipid peroxidation cascade initi-

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10.1021/jf990474l CCC: $18.00
Published on Web 10/06/1999

ated by the OH free radical (Quinlan et al., 1988; Oteiza, 1994), whereas glucose is a free radical scavenger (Kot et al., 1998). Protoplasting solubilizes bifidobacterial cell walls (Brigidi et al., 1986) while leaving bacterial membranes intact, and it is assumed in this work that the appearance of ^{59}Fe in the soluble fraction of protoplasted ^{59}Fe-laden bifidobacteria would represent iron bound to the cell walls. On the other hand, iron remaining on the protoplasts would represent membrane-bound iron.

B. thermophilum was used in this work as a model organism for the genus *Bifidobacterium* because this species is relatively easy to protoplast (Brigidi et al., 1986) and because of our extensive experience with this organism.

MATERIALS AND METHODS

Microorganism. *B. thermophilum* (ATCC 25866) was obtained from American Type Culture Collection (Rockville, MD). The organisms were grown in trypticase−proteose−yeast extract (TPY) medium (Scardovi, 1986) and were handled and tested as previously described (Kot and Bezkorovainy, 1998). In all manipulations, the cells were suspended in 0.1 M 3,3-dimethylglutarate buffer 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 of solution.

Protoplasting of *B. thermophilum* was carried out as previously described (Kot et al., 1993) using a mixture of lysozyme (Sigma Corp., St. Louis, MO, no. L-6876) and an *Aspergillus oryzae* protease (Sigma no. P-4755). The enzymes were present at 3 mg/mL concentration each, and the procedure was carried out for a total of 90 min. Protoplasts were further broken down into soluble and insoluble fractions by being suspended in water and ground in a glass homogenizer (Kot et al., 1993).

Iron-Binding Procedures. The $Fe(OH)_3$ system used for *B. thermophilum* iron-binding studies was designed to generate the maximum initial free radical effect. Because the Fenton reaction of $^{59}FeSO_4$ with H_2O_2 is a very rapid one and the OH free radical thus produced persists for a very short period of time (Halliwell and Gutteridge, 1985), the mixing of the two reactants was done in situ, that is, in the presence of the cells at 37 °C. Fe(III) was, of course, also produced by the Fenton reaction and became bound to the cells. The following illustrates the setup of a typical reaction mixture: 9.76 mL of the cell suspension (A_{610} = 2.4) in the 3,3-dimethylglutarate buffer at pH 6.5 and at 37 °C was mixed with 0.24 mL of 10 mM $^{59}FeSO_4$ in the same buffer at 37 °C. To this was added 10 mL of prewarmed 840 μM H_2O_2 in the pH 6.5 buffer. The final cell turbidity (A_{610}) was 1.2, and the $Fe(OH)_3$ concentration (following instantaneous oxidation of the ferrous iron by H_2O_2) was 120 μM. Appropriate dilutions of these reagents could be made to vary iron and H_2O_2 concentrations as desired. The H_2O_2 was always present in a 300 μM excess over that of iron. When desired, glucose (2 mg/mL) or 400 μM $AlCl_3 \cdot 6H_2O$ could be included in the reaction mixture.

This cell suspension (20 mL) was maintained at 37 °C with gentle shaking with 5-mL aliquots being withdrawn at the 5-, 30-, 60-, and 120 min marks. Each cell sample thus withdrawn was cooled in an ice bath, centrifuged in the cold, washed once with the pH 6.5 buffer, and resuspended in the protoplasting solution (Kot et al., 1993). Following the protoplasting procedure, the reaction mixture was centrifuged and the solubilized and protoplast fractions were counted.

The preceding iron-binding procedure was also carried out with different iron concentrations (30−120 μM), the cells were protoplasted, iron-binding results were obtained at various times, and iron concentrations extrapolated to zero time using the least-squares method. The zero time data were subjected to an analysis using Scatchard plots (Scatchard, 1949). This provided maximum iron-binding sites on both the cell walls and cell membranes at zero time, as well as dissociation constants. Alternately, whole cells were incubated with different $^{59}Fe/H_2O_2$ concentrations (iron = 30−120 μM) as described above for a total of 30 min, with samples being removed after 5, 10, 20, and 30 min of incubation. The cells were washed once with the pH 6.5 buffer solution and counted, and plots of Fe(III) bound versus time were constructed using the least-squares method. These were extrapolated to zero time, and the latter values were subjected to Scatchard analysis to determine the B_{max} and K_d.

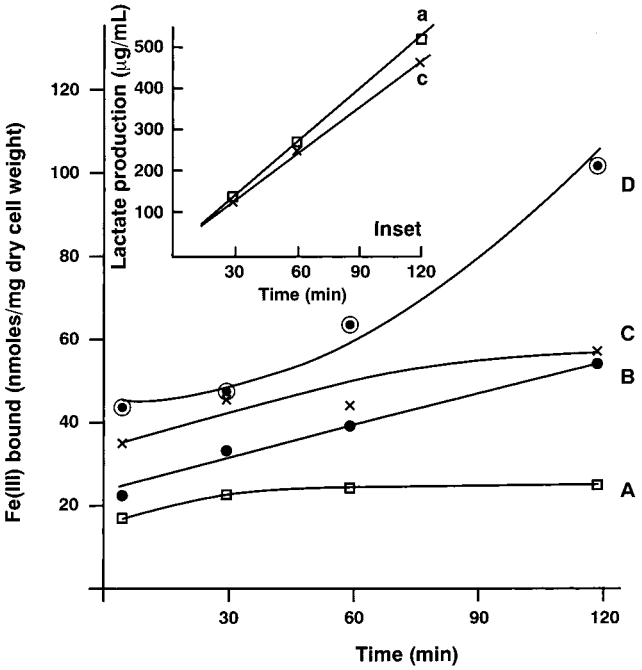

Figure 1. Binding of Fe(III) by *B. thermophilum* as a function of time in the presence of Al(III) and glucose: (curve A) cells in the presence of 2 mg/mL glucose and no Al(III); (curve B) cells without glucose or Al(III); (curve C) cells with 400 μM Al(III) and 2 mg/mL glucose; (curve D) cells with 400 μM Al(III) and no glucose. All iron concentrations were 100 μM. (Inset) (a) Lactate produced by cells represented by curve A; (c) lactate produced by cells represented by curve C.

Analytical Procedures and Materials. Hydrogen peroxide was determined colorimetrically using Sigma kit 352 as previously described (Kot and Bezkorovainy, 1998; Kot et al., 1997). Lactate analysis was done using Sigma kit 826 UV as previously described (Kot and Bezkorovainy, 1998). All colorimetric measurements were done with the Perkin-Elmer Lambda 2 spectrophotometer (Ueberlingen, Germany) and cell turbidity determinations were done in the Milton Roy Spectronic 21 instrument (Rochester, NY). The counting of ^{59}Fe was done in a Beckman Gamma-4000 counter (Palo Alto, CA).

$^{59}FeSO_4$ was obtained from DuPont Laboratories (Boston, MA), and $AlCl_3 \cdot 6H_2O$ was purchased from Fischer Scientific Co. (Fairview, NJ). All other chemicals, unless otherwise indicated, were bought from Sigma Corp.

RESULTS

Effect of Al(III) and Glucose on the Binding of Fe(III) to *B. thermophilum*. Figure 1 illustrates the binding of Fe(III) by *B. thermophilum* cells in the presence of glucose and/or Al(III). Cells represented by curve D were incubated with $Fe(OH)_3$ and Al(III) in the absence of glucose. These cells bound the most Fe(III). When the iron binding was carried out in the presence of Al(III) and glucose (curve C), the amount of Fe(III) bound was abated, although it was still higher than that in the absence of Al(III) (curves A and B). Curve B represents cells incubated with $Fe(OH)_3$ without Al(III) and glucose, whereas curve A represents cells incubated with $Fe(OH)_3$ in the presence of glucose, but not with Al(III). The latter bound the least amount of iron. Lactate production was not affected by Al(III) (inset, curve c).

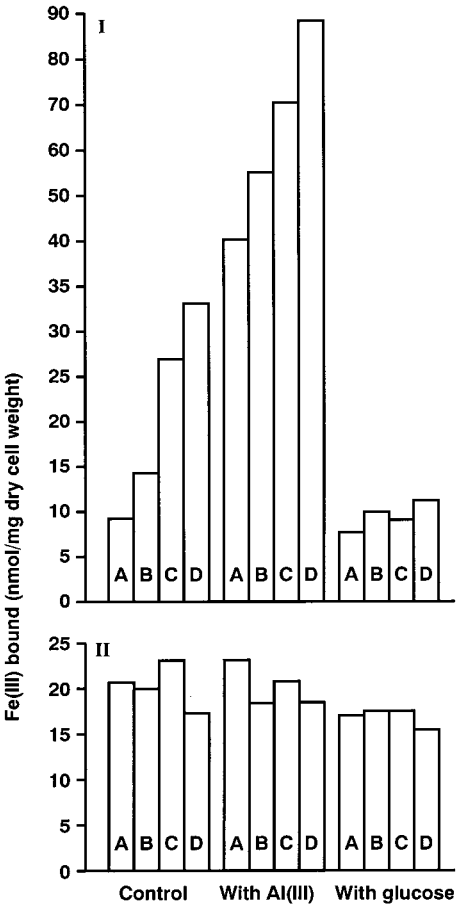

Figure 2. Binding of Fe(III) by cell walls and membranes of *B. thermophilum* following incubation with $Fe(OH)_3$ and then protoplasting: (I) cell membranes; (II) cell walls. Bars labeled A, B, C, and D refer to samples drawn after 5, 30, 60, and 120 min of incubation with $Fe(OH)_3$, respectively. Column series labeled "With Al(III)" refer to iron binding in the presence of 400 μM Al(III) and without glucose. Column series labeled "With glucose" refer to cells without Al(III) but in the presence of 2 mg/mL glucose. Column series labeled "Control" refer to cells incubated with $Fe(OH)_3$ in the absence of glucose and Al(III). $Fe(OH)_3$ concentration was 113 μM in all cases.

Protoplasting of Iron-Laden *B. thermophilum* Cells. Cells represented by curves A, B, and D in Figure 1 were subjected to protoplasting following incubation with 113 μM $Fe(OH)_3$ for 5, 30, 60, and 120 min. Following this procedure, both the supernatants and protoplasts were counted. The results are depicted in Figure 2; panel I represents the iron found in protoplasts, and panel II represents the iron solubilized by the protoplasting procedure (cell wall iron). Bars labeled "Controls" represented cells without Al(III) and glucose. The group labeled "With Al(III)" contained Al(III) but no glucose. The group labeled "With glucose" contained glucose but no Al(III).

There was no apparent difference between the control and "with Al(III)" cell wall groups; however, the group labeled "With glucose" had ~20% less iron than the preceding two groups (Figure 2, panel II). The protoplasts, on the other hand (Figure 2, panel I), showed increased iron binding with time in the cases of controls and "with Al(III)". The latter was especially heavy with iron. Protoplasts labeled "With glucose" contained small amounts of iron, and an increase with time was hardly visible.

Figure 3 indicates ratios of Fe(III) in protoplasts to those of respective soluble fractions (cell walls). Note that this ratio never reached 1 in case of the "with glucose" group (Figure 3, curve 3). In the case of the Al(III) group, this ratio was ~2 after 5 min of incubation with $Fe(OH)_3$ and rose to >5 after 120 min (Figure 3, curve 1). In the control group, a 1:1 ratio was achieved after about 1 h of incubation with $Fe(OH)_3$ (Figure 3, curve 2).

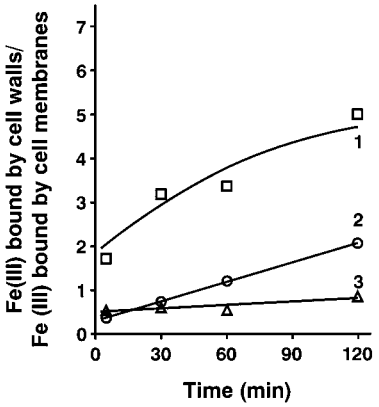

Figure 3. Cell membrane to cell wall iron ratios following the protoplasting of Fe(III)-laden *B. thermophilum* cells: (curve 1) cells with 400 μM Al(III) only; (curve 2) control cells [no glucose, no Al(III)]; (curve 3) cells with 2 mg/mL glucose only. All data are from Figure 2.

Protoplasts obtained from cells that had been incubated with $Fe(OH)_3$ for 120 min were subjected to an additional protoplasting procedure to ascertain whether the initial procedure had been complete. Little if any iron appeared in the supernatant following this second protoplasting protocol (data not shown). In addition, iron-laden protoplasts were in each case subjected to a lysis procedure and separated into soluble and insoluble fractions. Ninety percent of ^{59}Fe was recovered, 85% in the particulate fraction and 5% in the soluble fraction (details not shown).

Distribution of Iron in *B. thermophilum* Cells Incubated with Various $Fe(OH)_3$ Concentrations. Bifidobacteria were incubated with different $Fe(OH)_3$ concentrations without Al(III) or glucose and protoplasted, and Fe(III) content was determined in the cell wall and protoplast fractions. The results are depicted in Figure 4. It is again seen that the curves representing the solubilized (cell wall) portion were almost flat with time, whereas those representing protoplasts showed increased Fe(III) binding at all four iron concentrations. When the latter were extrapolated to zero time, the *y*-intercepts were close to zero, indicating that little if any iron was bound to the cell membranes instantaneously. On the other hand, extrapolation to zero time of curves representing Fe(III) bound to the cell walls gave *y*-intercepts of decreasing magnitude with decreasing $Fe(OH)_3$ concentrations. The data thus obtained were subjected to an analysis by the Scatchard method (1949), and the following results were obtained: maximum number of binding sites (B_{max}) 37.6 nmol/mg of dry cell weight and a dissociation constant (K_d) of 64.7 μM. Correlation coefficient for the Scatchard plot was 0.94. The corresponding B_{max} and K_d values for the protoplasts were 3.64 nmol/mg of dry cell weight (i.e., near zero) and 52.6 μM, respectively. Whole cells gave a B_{max} value of 33.3 nmol/mg of dry cell weight and a K_d of 41.1 μM.

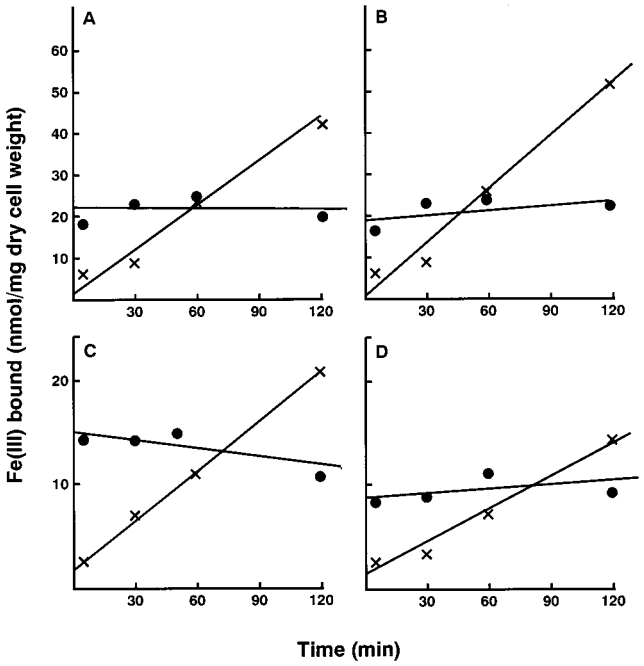

Figure 4. Distribution of Fe(III) on the surface of *B. thermophilum* as a function of time and $Fe(OH)_3$ concentration. Curves with solid circles represent cell walls; those with crosses represent cell membranes. Panels A, B, C, and D represent cells incubated with 118, 92.2, 52.6, and 30.0 μM $Fe(OH)_3$, respectively.

DISCUSSION

Our previous investigations have indicated that the binding of Fe(III) to bifidobacterial surfaces was facilitated, at least in part, by the action of free radicals (Bezkorovainy and Kot, 1998). Thus, a combination of iron (in either in the ferrous or ferric state) and H_2O_2 initiated free radical formation, which then proceeded to create Fe(III) binding sites on the bacterial surface. Our previous work, however, did not differentiate between the free radical-created iron-binding sites and those that may have been initially present on the bacterial surface. It is important to make this distinction to understand whether bifidobacteria can function as probiotics by withholding iron from pathogens under anaerobic conditions.

The work reported herein has utilized a method, based on the work of Richmond et al. (1981), of generating free radicals from Fe(II) and H_2O_2 that would be expected to produce a maximal effect. This reaction (Fenton reaction) results in the production of Fe(III) in the form of $Fe(OH)_3$ (Au-Yeung et al., 1985) and the OH free radical. The latter then initiates a lipid peroxidation cascade (Gutteridge, 1984). This cascade is enhanced by Al(III) (Quinlan et al., 1988; Oteiza, 1994), whereas the effects of the OH free radical can be abated by glucose (Bezkorovainy and Kot, 1998). The work reported herein has determined that Al(III) had enhanced Fe(III) binding by whole bifidobacterial cells, whereas glucose had abated it (Figure 1).

The protoplasting procedure used with *B. thermophilum* had apparently removed most if not all cell wall material, because little if any iron was released into the soluble fraction following a repeat of the protoplasting protocol. Such experiments indicated that iron was bound by both the cell walls and membranes. In the latter case, Fe(III) binding was the result of OH free radical-initiated lipid peroxidation effects, because Al(III) increased it and glucose abated it. In the case of cell walls, free radicals had a much weaker influence on Fe(III) binding, because Al(III) had no effect and glucose had a small (though discernible) negative influence. It may thus be concluded that Fe(III) binding to cell walls of *B. thermophilum* was affected by free radicals to only a minor extent. It was also obvious that in the case of cell walls, Fe(III) binding was almost instantaneous, because iron binding did not increase as a function of time (Figures 3 and 4). The zero time binding by the membranes was only $\sim 1/10$ that of cell walls, as shown by the Scatchard plots. With time, however, as the membrane lipid peroxidation cascade proceeded forth, more iron became bound. It should be noted that zero time Fe(III) binding by whole *B. thermophilum* cells showed B_{max} values of 33.3 nmol of Fe(III)/mg of dry cell weight. This compares well with the figure of 37.6 obtained for the cell wall fraction. The B_{max} value obtained for whole cells thus reflects largely the non-free radical binding of Fe(III) by the cell wall. The K_d values were, however, somewhat different, most likely reflecting a weakening of the Fe(III)−cell wall interaction following the protoplasting procedure.

Protoplasting experiments have thus demonstrated that bifidobacteria can bind iron [presented as $Fe(OH)_3$] in the absence of free radical action. The iron binds to the cell wall in such cases. On the other hand, maximum amounts of iron can be bound when free radical action is present, such as may exist locally in the intestinal tract at sites of inflammation, where white blood cells may be able to supply the H_2O_2 required for free radical effects (Ghio et al., 1997). Bifidobacteria can thus function as probiotics by limiting the amount of ferric iron available to pathogens as had been previously proposed (Bezkorovainy and Solberg, 1989) or as moderators of free radical-induced damage to intestinal tissue.

ACKNOWLEDGMENT

We thank Dr. Klaus Kuettner, Chairman, Department of Biochemistry, Rush Medical College, for supporting this project.

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Received for review May 7, 1999. Revised manuscript received July 6, 1999. Accepted August 31, 1999.

JF990474L