APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1992, p. 405–408
0099-2240/92/010405-04$02.00/0

Vol. 58, No. 1

Influence of Oxidation State on Iron Binding by *Bacillus licheniformis* Capsule

ROBERT J. C. McLEAN,[1,2]* DIANE BEAUCHEMIN,[3] AND TERRY J. BEVERIDGE[4]

Departments of Urology,[1] Microbiology and Immunology,[2] and Chemistry,[3] Queen's University, Kingston, Ontario, Canada K7L 3N6, and Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1[4]*

Received 22 August 1991/Accepted 28 October 1991

We examined ferric (Fe^{3+}) and ferrous (Fe^{2+}) iron binding by the anionic gamma-glutamyl capsule polymer of *Bacillus licheniformis* ATCC 9945. The addition of $FeCl_3$ to *B. licheniformis* capsule under aerobic conditions resulted in flocculation due to the capsule-induced formation of amorphous, rust-colored ferrihydrite. Significant binding of iron, which could be attributed to binding by both the anionic capsule and the ferrihydrite precipitate, occurred. In contrast, the addition of $FeCl_2$ to *B. licheniformis* capsule under anaerobic conditions resulted in significantly less iron being bound and no color change or flocculation occurring. Capsule-bound ferric iron could be partially released upon addition of several reducing agents. From these observations, it can be concluded that the oxidation state of iron significantly influences its tendency to be bound by anionic bacterial polymers such as capsules.

Metal binding by anionic bacterial surface structures is a well-documented phenomenon having notable biological and environmental implications (1, 2, 4, 6, 12). Traditionally, most studies of metal binding by bacterial structures have consisted of suspending these materials in chemically defined solutions of known metal composition and measuring metal binding (reviewed in references 2 and 12). Yet, in their normal habitats the natural physiological processes of living bacteria greatly complicate metal binding. One significant effect of bacterial metabolism is oxygen depletion and generation of anaerobic reducing conditions (8). Significant quantities of mobile metals are often found in reducing environments such as sediments, and sorption rates arising from bacterial activity can differ from more oxygenic horizons. This study was undertaken to investigate the effects of altered redox conditions on iron binding by the well-characterized gamma-glutamyl capsule polymer of *Bacillus licheniformis* (11, 16).

(A preliminary report of this work was presented previously [13].)

B. licheniformis ATCC 9945 was originally obtained from F. A. Troy, Department of Biological Chemistry, University of California, Davis, and stored in the culture collection of the Department of Microbiology, University of Guelph. Procedures for capsule production and purification were described in a previous publication (11).

The experimental protocol for metal binding involved suspending lyophilized capsule (1 mg ml^{-1}) in a solution containing $FeCl_3$ or $FeCl_2$. After 1 h of incubation, unbound metal was removed by dialysis and the capsule-metal mixture was lyophilized and analyzed for metal content by inductively coupled plasma-mass spectrometry or atomic absorption spectroscopy as described elsewhere (11). The major modification in the present study was that all binding experiments involving ferrous iron were carried out under anaerobic conditions with an anaerobic glove box containing a 5% CO_2–10% H_2–85% N_2 atmosphere (3). All water used was high-resistance (18-MΩ cm^{-1}) water (HRW) (pH 3.8 to 4.5). HRW used for anaerobic metal binding (including dialysis) was dispensed into 500-ml nitric acid-leached polycarbonate containers and left in the anaerobic chamber overnight at room temperature (23°C) to remove dissolved oxygen. The metal-free dialysis tubing (12- to 14-kDa exclusion) used in these experiments was washed five times in HRW and also left standing overnight in HRW in the anaerobic chamber. All solutions for ferrous iron binding were freshly prepared under anaerobic conditions before use.

Since *B. licheniformis* capsule contains 8.2 μmol of anionic COO^- sites per mg of capsule (11), iron concentrations were set to be the ionic equivalent of 50, 100, or 200% of the available sites. In the case of Fe^{2+}, the relevant concentrations used were 2.05, 4.1, and 8.2 μmol mg^{-1}. Fe^{3+} binding was reported previously (11). Fe^{2+} binding involved suspending ca. 10 mg of *B. licheniformis* capsule in a freshly prepared solution of $FeCl_2$ (1 mg of capsule ml^{-1}). After 1 h of equilibration at room temperature, the capsule-metal mixture was placed into metal-free, sulfur-free dialysis tubing under anaerobic conditions. Samples were then removed from the glove box, placed into tared plastic containers, immediately frozen with liquid nitrogen (to reduce the risk of oxidation), lyophilized, weighed to within 0.01 mg, and suspended in 7.5 M HNO_3 (1 mg ml^{-1}) to totally dissolve the polymer and release all bound metal prior to metal analysis. All metal-binding experiments were performed in triplicate.

To examine the effects of oxidation state on the stability of capsule-bound ferric iron, we suspended ca. 10 mg of *B. licheniformis* capsule (1 mg ml^{-1}) in 2.73 mM $FeCl_3$ under aerobic conditions. This concentration of Fe^{3+} was equivalent to 100% ionic saturation of the capsule (11). Following 1 h of equilibration at room temperature, a reducing agent was added to the capsule-ferric iron mixture. Three different substances were chosen so as to employ a carbon-based, a sulfur-based, and a metal-based reducing agent. These were, respectively, ascorbic acid (1.0% [wt/vol] stock solution) (14), cysteine-HCl (2.5% [wt/vol] stock solution) (15), and titanium(III) citrate (stock solution contained 5 ml of 15% titanium trichloride solution plus 50 ml of 0.2 M sodium citrate) (3). The reducing-agent solutions were added to the

* Corresponding author.

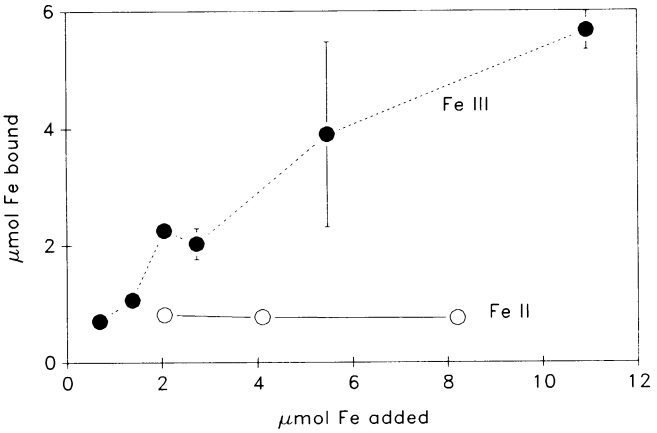

FIG. 1. Ferric (Fe^{3+}) and ferrous (Fe^{2+}) binding by *B. licheniformis* capsule. Fe^{3+} results were reported previously (11) and are reprinted with permission of the American Society for Microbiology.

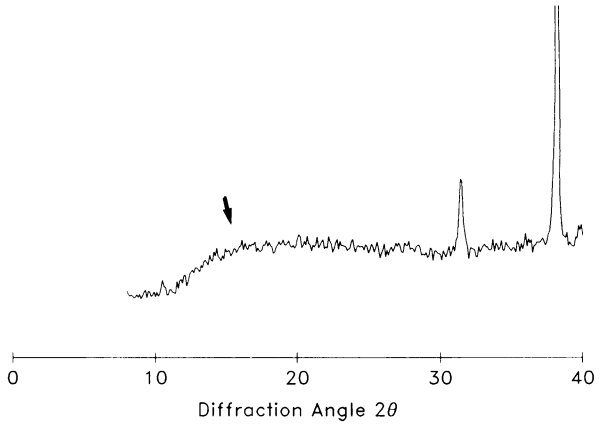

FIG. 2. X-ray diffraction of amorphous ferric hydroxide forming as a consequence of aerobic Fe^{3+} binding by *B. licheniformis* capsule. The peaks are due to the aluminum sample holder. The broad amorphous hump indicating the unordered structure of the ferric hydroxide is indicated by the arrow.

capsule-iron mixture in the following volumes: ascorbic acid, 350 μl/10 ml; cysteine-HCl, 400 μl/10 ml; and titanium(III) citrate, 300 μl/10 ml. A fourth experimental group of capsule-iron mixture did not have reducing agent added. After the addition of reducing agent, the caps on all vials were loosened to facilitate gas exchange, and the mixture was incubated for 1 week at room temperature under either aerobic or anaerobic conditions. Following incubation, unbound metal was removed by dialysis under either aerobic or anaerobic conditions and processed for trace metal analysis. These remobilization experiments were performed in triplicate for trace metal analysis. A fourth replicate was also performed so that, following incubation, aliquots could be removed and tested for pH, E_h, and ferrous iron concentration (described below). Metal contamination was monitored in all experiments by the use of process controls (11). All samples were tested for metal content by either atomic absorption spectroscopy or inductively coupled plasma-mass spectroscopy using the protocols described elsewhere (11). Fe^{2+} concentrations were measured by the ferrozine colorimetric assay (8). Powder X-ray diffraction analysis of the ferric hydroxide precipitates was performed as described previously (11).

pH measurements were taken before and immediately after metal binding. Redox (E_h) measurements were performed with an Orion model 601A digital Ionalyzer fitted with a redox combination electrode (Orion Research Inc., Boston, Mass.). Uncorrected E_h measurements were taken with the internal calomel reference electrode as an internal standard as outlined in the manufacturer's instructions.

Fe^{3+} and Fe^{2+} binding to *B. licheniformis* capsule is shown in Fig. 1. Fe^{3+} binding resulted in the immediate formation of rust-colored ferric hydroxide (ferrihydrite) and polymer flocculation. Flocculation due to Fe^{3+} occurred under both aerobic and anaerobic conditions. X-ray diffraction of this ferrihydrite precipitate showed it to be amorphous and to lack any ordered structure (Fig. 2). In contrast, Fe^{2+} binding under anaerobic conditions did not produce any color change, nor did it induce mineral precipitation or flocculation. Incubation of Fe^{2+} with capsule under aerobic conditions resulted in rust-colored ferrihydrite flocculation after 5 to 10 min due to oxidation of Fe^{2+} to Fe^{3+}. No Fe^{3+} precipitation occurred in the absence of capsule (11).

Suspension of *B. licheniformis* capsule (1 mg ml^{-1}) for 1 h under aerobic conditions in 2.73 mM $FeCl_3$ (equivalent to 100% ionic saturation [11]) resulted in the binding of 2.030 ± 0.156 μmol of Fe per mg. Subsequent addition of the reducing agents ascorbic acid, cysteine-HCl, and titanium(III) citrate to this mixture and incubation for 1 week resulted in less iron remaining bound (Fig. 3). Incubation of the Fe(III)-capsule mixture in the absence of reducing agent did not significantly affect the quantity of iron bound (Fig. 3). Addition of reducing agents lowered the E_h, slightly affected the pH, and elevated Fe^{2+} concentrations (Table 1). Rust-colored ferrihydrite, initially present, disappeared after 2 or 3 days in samples incubated anaerobically with reducing agents. In samples incubated aerobically, the visible quantity of ferrihydrite diminished but did not disappear. No visible change in the ferrihydrite occurred in samples lacking a reducing agent. The differences between Fe^{2+} concentrations in samples incubated aerobically and anaerobically in

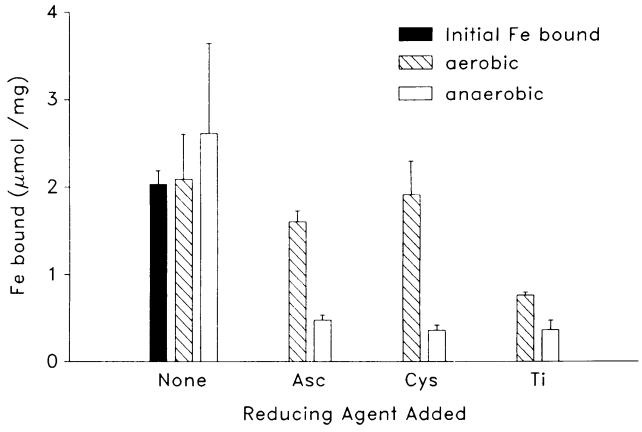

FIG. 3. Remobilization of capsule-bound Fe^{3+} due to chemical reduction. Fe^{3+} was bound to capsule. Reducing agents [None, no reducing agent; Asc, ascorbic acid; Cys, cysteine-HCl; Ti, titanium(III) citrate] were then added, and the mixture was incubated for 1 week under aerobic or anaerobic conditions. For details, please refer to the text.

TABLE 1. pH, E_h, and soluble Fe^{2+} concentrations of *B. licheniformis* capsule in Fe^{3+} mixtures before and after 1 week of incubation with reducing agents

Reducing agent used	Results with:					
	Aerobic incubation			Anaerobic incubation[a]		
	pH	E_h (mV)	Fe^{2+} concn (mM)	pH	E_h (mV)	Fe^{2+} concn (mM)
2.73 mM $FeCl_3$ solution[b]	2.70	571	0	NA	NA	NA
Capsule-Fe^{3+} before 1-week incubation	2.76	583	0	NA	NA	NA
None	2.83	483	0.061	2.79	491	0.225
Ascorbic acid	3.08	310	1.08	3.39	333	1.38
Cysteine-HCl	2.69	280	1.22	2.77	291	2.56
Titanium(III) citrate	2.08	337	2.57	2.10	342	2.46

[a] NA, not applicable.
[b] Solution in which capsule was initially suspended.

the presence of reducing agents likely reflect both their limited exposure to oxygen and the action of the various reducing agents.

Dissolved iron in aqueous environments is quite unstable. Under aerobic conditions and a pH of >5, Fe^{3+} is a transient species and, depending upon its chemical environment, tends to precipitate as amorphous or crystalline mineral forms. If OH^- is readily available, ferric hydroxide and ferric oxide are formed (7). When present, anionic structures such as *Bacillus subtilis* walls, *B. licheniformis* capsules, and clays promote ferric hydroxide formation (10, 17). At a pH of >3, Fe^{2+} can spontaneously oxidize to Fe^{3+} (7, 9). Because of this instability, all solutions containing iron were freshly prepared and used within 10 min of preparation. In addition, Fe^{2+} solutions were prepared with degassed HRW under anaerobic conditions. In solutions, both Fe^{2+} and Fe^{3+} form hexahydrated aquo-ions having the general formulae of $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$, respectively. The sizes of these two hydrated ions are similar, although the ferric aquo-ion, by virtue of its greater charge density, is slightly smaller (9). On the basis of coordination chemistry and ionic size alone, one would not expect to encounter significant differences in the binding characteristics of these two ions by the carboxylate moieties of the capsule. The notable differences evident in the binding of ferric and ferrous iron by *B. licheniformis* capsule (Fig. 1) are due to the greater charge density of the ferric ion, the spontaneous formation of ferrihydrite during Fe^{3+} binding to capsule, and the tendency of this mineral to bind additional metal (5, 11).

A number of factors influence iron reduction. Amorphous iron is significantly more prone to microbial reductive processes than crystalline iron is (7). All ferrihydrite flocs produced in our experiment were amorphous (Fig. 2) with no evidence of any ordered (crystalline) structure. The reducing agents used for remobilization might also influence iron binding. Their potential effects would include chelation [citrate in titanium(III) citrate], complex formation with cysteine or ascorbate, and competition for metal-binding sites by Ti^{3+} or Ti^{4+}. Because of these potential interactions, we selected three chemically different reducing agents. In all cases the addition of a reducing agent lowered the E_h, induced the formation of Fe^{2+} (Table 1), and resulted in similar but smaller quantities of iron being retained (Fig. 3).

We conclude that iron binding by the anionic gamma-glutamyl capsule polymer of *B. licheniformis* is significantly influenced by the oxidation state of iron. Less Fe^{2+} than Fe^{3+} is bound, and bound Fe^{3+} can be released from the capsule by chemical (and conceivably biological) reduction. In nature, other metals which bind to bacterial surface polymers through ferrihydrite (2, 10, 12) would also be expected to remobilize upon its reduction. Microbial binding and remobilization of other metals having several oxidation states (Mn, Cr, and Hg, etc.) may also be similarly affected. The environmental implications of redox-sensitive metal binding in anaerobic, metal-rich environments are therefore quite significant.

This project was initiated while R.J.C.M. was supported by a fellowship from the Alberta Heritage Foundation for Medical Research during his tenure as a postdoctoral fellow in T.J.B.'s laboratory. Funding for this study was provided by operating grants from the Natural Sciences and Engineering Research Council of Canada to R.J.C.M., D.B., and T.J.B. R.J.C.M. is funded by a Career Scientist Fellowship from the Ontario Ministry of Health.

We express our appreciation to L. Clapham, Department of Physics, Queen's University, for the X-ray diffraction analysis. We also thank C. W. Forsberg (University of Guelph) and K. F. Jarrell and A. Kropinski (Queen's University) for the use of their anaerobic chambers, A. Ley (Ontario Ministry of the Environment, Kingston, Ontario, Canada) for the use of his equipment for E_h measurements, and two anonymous referees for their helpful comments.

REFERENCES

1. **Babich, H., and G. Stotzky.** 1980. Environmental factors that influence the toxicity of heavy metal and gaseous pollutants to microorganisms. Crit. Rev. Microbiol. **8**:99–145.
2. **Beveridge, T. J.** 1989. Role of cellular design in bacterial metal accumulation and mineralization. Annu. Rev. Microbiol. **43**: 147–171.
3. **Costilow, R. N.** 1981. Biophysical factors in growth, p. 66–78. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
4. **Ferris, F. G., S. Schultze, T. C. Witten, W. S. Fyfe, and T. J. Beveridge.** 1989. Metal interactions with microbial biofilms in acidic and neutral pH environments. Appl. Environ. Microbiol. **55**:1249–1257.
5. **Frimmel, F. H., and J. Geywitz.** 1987. Direct polarographic recording of metal elimination from aquatic samples by coprecipitation with ferric hydroxide. Sci. Total Environ. **60**:57–65.
6. **Geesey, G. G., and L. Jang.** 1990. Extracellular polymers for metal binding, p. 223–247. *In* H. L. Ehrlich and C. L. Brierley (ed.), Microbial mineral recovery. McGraw-Hill Book Co., New York.
7. **Lovley, D. R.** 1987. Organic matter mineralization with the reduction of ferric iron: a review. Geomicrobiol. J. **5**:375–399.
8. **Lovley, D. R., and E. J. P. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51**:683–689.
9. **Magini, M.** 1988. X-ray diffraction of ions in aqueous solutions: hydration and complex formation, p. 100–102. CRC Press, Inc., Boca Raton, Fla.
10. **Mayers, I. T., and T. J. Beveridge.** 1989. The sorption of metals to *Bacillus subtilis* walls from dilute solutions and simulated Hamilton Harbour (Lake Ontario) water. Can. J. Microbiol. **35**:764–770.
11. **McLean, R. J. C., D. Beauchemin, L. Clapham, and T. J. Beveridge.** 1990. Metal-binding characteristics of the gamma-glutamyl capsule polymer of *Bacillus licheniformis* ATCC 9945. Appl. Environ. Microbiol. **56**:3671–3677.
12. **McLean, R. J. C., and T. J. Beveridge.** 1990. Metal binding capacity of bacterial surfaces and their ability to form mineralized aggregates, p. 185–222. *In* H. L. Ehrlich and C. L. Brierley (ed.), Microbial mineral recovery. McGraw-Hill Book Co., New York.

13. **McLean, R. J. C., C. A. Flemming, S. G. Walker, and T. J. Beveridge.** 1988. Influence of oxidation state on metal ion binding affinity to bacterial surface components, abstr. EM6. Can. Soc. Microbiol. Annu. Meet. Abstr., vol. 38.
14. **Neal, J. L., Jr., and R. I. Larson.** 1976. Acetylene reduction by bacteria isolated from the rhizosphere of wheat. Soil Biol. Biochem. **8**:151–155.
15. **Scott, H. W., and B. A. Dehority.** 1965. Vitamin requirements of several cellulolytic rumen bacteria. J. Bacteriol. **89**:1169–1175.
16. **Troy, F. A.** 1973. Chemistry and biosynthesis of the poly (γ-D-glutamyl) capsule in *Bacillus licheniformis*. J. Biol. Chem. **248**:305–315.
17. **Walker, S. G., C. A. Flemming, F. G. Ferris, T. J. Beveridge, and G. W. Bailey.** 1989. Physicochemical interaction of *Escherichia coli* cell envelopes and *Bacillus subtilis* cell walls with two clays and ability of the composite to immobilize heavy metal from solution. Appl. Environ. Microbiol. **55**:2976–2984.