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Inability to detect mycobactin in Mycobacteria-infected tissues suggests an alternative iron acquisition mechanism by Mycobacteria *in vivo*

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Although most species of mycobacterium are capable of producing mycobactin, it is not known if conditions within the host allow for mycobactin synthesis or whether it even plays a role in iron acquisition *in vivo*. We employed the mycobactin-auxotroph, *Mycobacterium paratuberculosis*, in a bioassay to examine tissues from animals infected with either *Mycobacterium tuberculosis*, *Mycobacterium avium* or *M. paratuberculosis* for the presence of mycobactin or compounds which demonstrate mycobactin-like activity. Other iron-binding compounds, including purified siderophores from unrelated organisms and host iron-binding proteins were also evaluated in the bioassay for growth induction of *M. paratuberculosis* in the absence of mycobactin. Although mycobactin could be easily demonstrated in tissues artificially seeded with mycobacteria, no mycobactin could be detected in heavily infected tissues. None of the purified siderophores from unrelated microorganisms were found to support growth of *M. paratuberculosis* in the absence of mycobactin. Host iron-binding proteins (transferrin, lactoferrin, ferritin, hemin) also failed to induce growth in the bioassay at pH 6.8, however, when the pH was adjusted between 5–6.2, transferrin and lactoferrin promoted growth of *M. paratuberculosis* without mycobactin, probably as a result of the dissociation of iron rather than a specific interaction. We confirm that mycobacteria are incapable of iron uptake when iron is chelated to siderophores from unrelated organisms and conclude that mycobactin-mediated mechanisms of iron-acquisition by mycobacteria do not appear to have as significant a role *in vivo* as *in vitro*. In addition, evidence is presented that suggests iron-containing transferrin and lactoferrin at low pH may circumvent the need for mycobactin by *M. paratuberculosis*.

Key words: mycobactin; siderophores; mycobacteria; paratuberculosis; iron; transferrin; lactoferrin.

Introduction

There is abundant evidence suggesting that iron occupies a central role in the ability of a microbe to infect and cause disease within a host.[1] It is generally assumed that, in the host, iron is tightly bound to specific host iron-binding proteins[2] and unavailable

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for bacterial consumption. Many bacteria have consequently evolved high affinity mechanisms for securing iron within the restrictive environment of the host.[3] Microbial synthesis of low-molecular-weight iron-chelating compounds, collectively known as siderophores, help overcome iron-deprivation in the environment by virtue of their strong affinity for iron.[4] Numerous enteric bacteria have even evolved specific mechanisms for utilizing siderophores synthesized by unrelated organisms, thus underscoring the importance of securing iron.[5–7]

Mycobacteria, like most bacteria, require iron for growth. Most mycobacterial species, with the exception of *Mycobacterium paratuberculosis* and related strains, produce cell wall-associated siderophore-like compounds known as mycobactins.[8,9] In addition, a second group of low-molecular-weight mycobacterial siderophores termed exochelins have been isolated from *Mycobacterium tuberculosis* and other mycobacterium species.[10] Production of mycobactin and exochelin by mycobacteria is regulated by the concentration of iron in the environment, being synthesized in greater amounts following iron-deficient growth.[11] Several mechanisms of iron uptake involving mycobactins and exochelins have been proposed, however, the role of each *in vivo* remains uncertain.[12]

M. paratuberculosis is the causative agent of Johne's disease, a chronic inflammatory bowel disease of ruminants, and has been isolated from patients with Crohn's disease.[13] Growth of *M. paratuberculosis* requires mycobactin added exogenously to culture media, but it is not known how these mycobactin-dependent mycobacteria acquire iron for growth within the host. Mycobacteria are facultative intracellular pathogens which survive and multiply within host macrophages. The most likely source of iron *in vivo* would appear to be that which is bound to host iron-binding proteins. A number of bacterial pathogens have been shown to acquire iron from host proteins by multiple mechanisms.[14–16] Reports that transferrin and lactoferrin are found in increased amounts in the lesions of animals with paratuberculosis[17] and that siderotic macrophages support the survival and multiplication of *M. paratuberculosis* better than macrophages with little or no iron[18] suggest that host iron-binding proteins might play a role in iron acquisition by mycobacteria *in vivo*.

The purpose of this study was to examine whether pathogenic mycobacteria synthesize detectable levels of mycobactin *in vivo* and to investigate the ability of *M. paratuberculosis* to utilize various iron-binding compounds as sources of iron.

Results

Bioassay demonstrating mycobactin-dependence of M. paratuberculosis

Growth of *M. paratuberculosis* was considered to have occurred when the cumulative growth units (1 unit = 0.25 nCi ^{14}C) reached 30. Cumulative growth units $\leqslant 30$ after 3 weeks of incubation were interpreted as no growth. Following this criteria, growth of *M. paratuberculosis* in the presence of 1.2 μM mycobactin J was observed by day 12. Neither strain of *M. paratuberculosis* was able to grow in 7H12B medium (pH 6.8) without the exogenous addition of mycobactin, owing to the inability to assimilate iron. The concentration of iron in 7H12B medium was calculated and measured to be approximately 140 μM, primarily as ferric ammonium citrate. The sensitivity of the bioassay for mycobactin was determined to be 6 nM.

Purified siderophores from unrelated organisms (enterobactin, ferrichrome, ferrichrome A, rhodorulate, aerobactin and multocidin) represented six different major structural families of iron-binding compounds and were tested in their iron-bound forms in concentrations ranging from 1 μM to 100 μM for growth promotion of *M. paratuberculosis*. None of the purified siderophores tested were capable of supporting

growth of *M. paratuberculosis* in the absence of mycobactin. In addition, growth was not induced with the common catechol, 2,3-dihydroxybenzoic acid (DHBA) nor when salicylate, a precursor of mycobactin, was added in concentrations up to 100 μM. Attempts to procure or make our own exochelins for evaluation in the bioassay were unsuccessful.

Host proteins which bind iron were examined for their ability to serve as sources of iron for *M. paratuberculosis*. Transferrin, lactoferrin and ferritin were added to the bioassay medium (pH 6.8) as the only source of iron in final concentrations of 10 μM and 100 μM at iron saturation levels of 25%, 50% and 100%. Iron-saturated hemin and hemoglobin were tested in final concentrations of 300 μM and 735 μM, respectively. Regardless of the concentration of iron-binding protein or the level of iron-saturation, no iron-binding protein was able to support growth of *M. paratuberculosis* at pH 6.8 unless mycobactin was also added.

Bioassay for mycobactin in tissues containing Mycobacteria

Extracts of all tissues seeded with non-pathogenic *Mycobacterium phlei* or *Mycobacterium smegmatis* contained mycobactin-like activity as demonstrated by the bioassay (Table 1). We will refer to induction of growth in the bioassay as a result of mycobactin activity since no other compound has been shown to circumvent the need for mycobactin. Bioassay growth was comparable to extracts of tissue containing 40 μg mycobactin J per gram tissue and validated the capacity of the extraction technique to recover mycobactin from tissue homogenates.

Naturally infected bovine ileum (*M. paratuberculosis* UWIS B025) and flamingo liver (*Mycobacterium avium* SDZOO) were homogenized and the number of cfu/g tissue determined to be $>10^{7.2}$ and $>10,^{8.3}$ respectively. It is important to note that the determination of cfu/g of tissue probably underestimates the total number of mycobacteria, as most were contained in large aggregates and many may not have been viable. Attempts to extract mycobactin from these naturally infected tissues with chloroform were unsuccessful as no mycobactin-like activity could be demonstrated in these tissues (Table 2). Likewise, chloroform extracts of pooled spleen homogenates (24 g) from mice experimentally infected with *M. tuberculosis* did not contain any demonstrable mycobactin.

M. avium SDZOO was subsequently cloned on low-iron chemically defined medium (CDM) and the harvested cells were seeded into sterile bovine liver homogenate taking precautions to maintain the same relative concentration of bacteria as in the original flamingo liver. The seeded liver homogenate was extracted with chloroform as previously described and tested for mycobactin in the bioassay whereby growth of

Table 1 Mycobactin activity in tissues seeded with mycobacteria

Species seeded[a]	cfu/g tissue[b]	Mycobactin activity detected in tissues[c]
None	—	None
M. smegmatis	6.8–7.6	+
M. phlei	7.9–9.0	+
M. avium SDZOO	8.3–9.0	+

[a] Strains were iron starved prior to seeding tissues.
[b] Range determined from duplicate dilution plate count data and is expressed as $\log_{10}$ values. Tissues are bovine liver and colon.
[c] Mycobactin activity determined by bioassay.

Table 2 Mycobactin activity in tissues from animals infected with mycobacteria

Species	cfu/g tissue[a]	Tissue sources[b]	Mycobactin activity in tissues[c]
M. paratuberculosis UWIS B025	7.2–8.0	Bovine ileum and colon	—
M. avium SDZOO	7.8–8.9	Flamingo spleen and liver	—
M. tuberculosis	4.0–5.2	Mice spleen	—

[a] Determined from duplicate dilution plate count data expressed as $\log_{10}$ values.
[b] Bovine and flamingo tissues were from naturally infected animals. The mice were experimentally infected.
[c] Mycobactin activity determined by bioassay.

M. paratuberculosis occurred in as little as 18 days and provided evidence of the capability of mycobactin production by *M. avium* SDZOO (Table 1).

Influence of pH on growth kinetics of M. paratuberculosis *in 7H12B medium*
Earlier work showed that *M. paratuberculosis* can grow in the absence of mycobactin when the iron concentration is above a minimum level dependent upon pH.[19] As the pH of the medium is reduced, the amount of iron required for growth is also lowered. Addition of Desferal® (200 μM) sufficiently sequestered iron in 7H12B medium making it unavailable for assimilation by *M. paratuberculosis* resulting in bacteriostasis (Fig. 1). The subsequent addition of iron (400 μM ferric ammonium citrate) back to the deferrated medium reversed the effect of bacteriostasis and promoted growth of *M. paratuberculosis*. Since iron was added back in excess, limited growth of *M. paratuberculosis* also occurred at pH 6.8 in the absence of mycobactin. Addition of ferrimycobactin J (1.2 μM) to the deferrated medium stimulated growth of *M. paratuberculosis* to a greater extent and over a wider range of pH. Similar findings were also demonstrated for strain UWIS B025 (data not shown), however, growth was not as good in the deferrated medium. Our experience with clinical isolates show

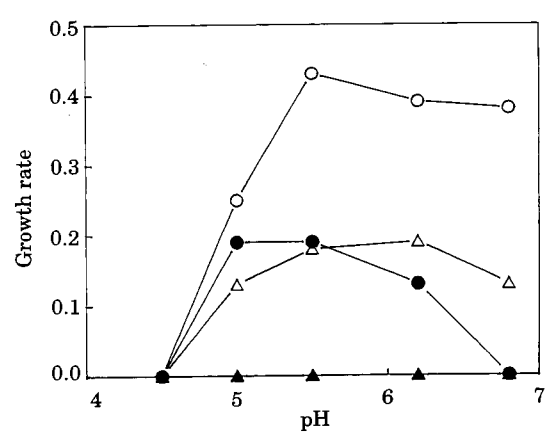

Fig. 1. Growth kinetics of *M. paratuberculosis* ATCC 19698 in 7H12B medium without mycobactin adjusted to different pHs. Additions to 7H12B medium: none (●), 0.2 mM desferal; (▲), 0.2 mM desferal and 400 μM ferric ammonium citrate (FAC); (△), 0.2 mM desferal and 1.2 μM mycobactin (○). Each point represents the mean of duplicate determinations. The coefficient of variation was <0.01 for all values.

that they grow more poorly in 7H12B medium. Growth rates of 0.3, 0.2, 0.1 correspond to generation times of approximately 2.3, 3.5 and 6.9 days, respectively, for *M. paratuberculosis* ATCC 19698.

Influence of iron-binding proteins on growth of M. paratuberculosis *in deferrated 7H12B medium at various pH*

Because *M. paratuberculosis* grew without mycobactin at low pH when moderate concentrations of iron in the medium were added, we examined iron-binding proteins which would have relevance to the *in vivo* environment and perhaps serve as a source of iron. *M. paratuberculosis* ATCC 19698 and UWIS B025 were tested in deferrated 7H12B medium for their ability to assimilate iron from either 50% iron-saturated 200 μM transferrin, lactoferrin or 20 μM 100% iron-saturated ferritin at lower pH. No growth was seen at any pH when ferritin was the sole source of iron (Fig. 2). Ferric-transferrin added to deferrated medium at pH 5.0 and 6.2 stimulated growth of *M. paratuberculosis* which was comparable to control vials containing ferric ammonium citrate. Growth in transferrin-containing medium at pH >6.2, however, was greatly reduced. Presumably at higher pH, iron was more tightly bound to transferrin, was less soluble or both. In contrast, lactoferrin also supported growth but only minimally and only at the extreme lower pH range.

Dissociation of iron from host iron-binding proteins as a function of pH has been well studied, however, we measured the relative amount of iron which dissociates from each of the host proteins as a function of pH in radiometric CDM. The high affinity of these proteins for iron resulted in their slow dissociation from iron which was measured spectrophotometrically. Iron bound to bovine transferrin readily became dissociated at pH $\leqslant 6.5$ and no transferrin-bound iron could be detected at pH 4.5 (Fig. 3). Lactoferrin also became dissociated from iron with decreasing pH but the degree of dissociation was slower and much less than that observed for transferrin. Even at pH 3.5, greater than 25% of lactoferrin was still bound to iron. No dissociation of iron from ferritin was observed until the pH was lowered below 4.3. The amount of free iron released from transferrin was directly related to the growth rate of *M. paratuberculosis*.

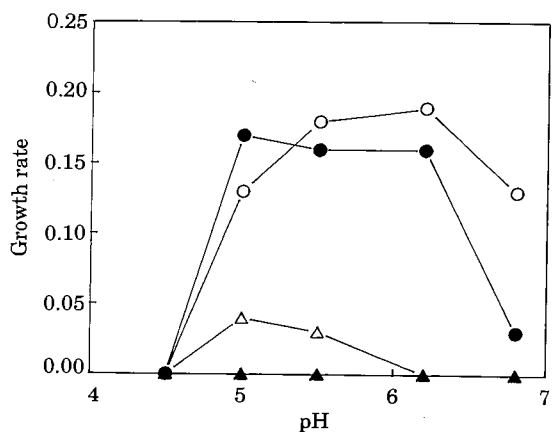

Fig. 2. Growth rate of *M. paratuberculosis* ATCC 19698 in deferrated 7H12B medium supplemented with either 400 μM ferric ammonium citrate (FAC) (○), 200 μM 50% iron-saturated transferrin (●), 200 μM 50% iron-saturated lactoferrin (△), or 20 μM iron-saturated ferritin (▲). The supplemented media was adjusted to pH between 4.5 and 6.8. All points represent the mean of duplicate determinations (CV <0.01).

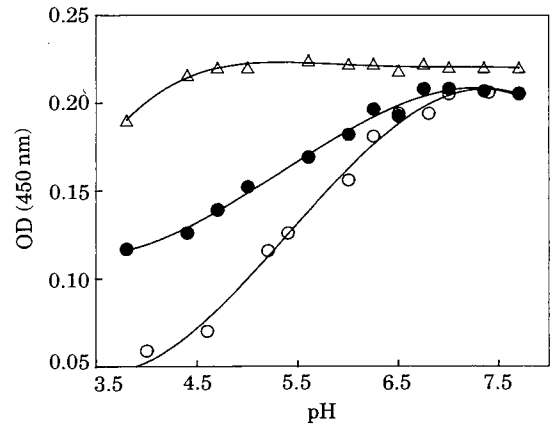

Fig. 3. Effect of pH on the dissociation of iron from 50% iron-saturated transferrin (○), 50% iron-saturated lactoferrin (●), and 100% iron-saturated ferritin (△) in radiometric chemically defined medium.

Discussion

The objectives of this study were to determine the response of *M. paratuberculosis* to exogenous siderophores and iron-binding proteins in the absence of mycobactin and to ascertain whether pathogenic mycobacterium synthesize detectable amounts of mycobactin *in vivo*. Since *M. paratuberculosis* requires exogenously added mycobactin for growth *in vitro*, it follows that for this organism to survive and grow *in vivo* either: (i) conditions within the host must allow for mycobactin synthesis; (ii) there is a co-infection with a mycobactin-producing species; or (iii) another mechanism, independent of mycobactin, is operative. Because bioassays are orders of magnitude more sensitive than chemical assays for the detection of siderophores, we chose a radiometric bioassay, employing the mycobactin-dependent species, *M. paratuberculosis*, for detection of mycobactin.

M. paratuberculosis is an intestinal pathogen and since the host intestine is a heterogenous competitive environment of assorted microbes it seemed possible that interaction with unrelated microorganisms or their siderophores might represent a potential mechanism for acquisition of iron. While a survey of all known siderophores would not have been practical, it seemed worthwhile to examine those siderophore compounds which are common and representative of different classes of chemical structures, or are known to be exploited by taxonomically dissimilar groups of microbes. Although mycobactins can satisfy the growth requirement of other selected siderophore-auxotrophs, only mycobactins, exochelins and the closely related nocobactins have so far been shown to satisfy the iron growth requirement of *M. paratuberculosis*. None of the purified siderophores, nor the iron-binding compounds extracted from colon contents were able to circumvent the need for mycobactin by *M. paratuberculosis*. Thus, these data support previous reports that only mycobactins and exochelins can satisfy the *in vitro* growth requirement of *M. paratuberculosis* for iron at pH 6.8.

The lack of detectable mycobactin in tissues heavily infected with pathogenic mycobacteria suggests that mycobactin may not be absolutely required for iron acquisition by mycobacteria within the host. We were unable to detect mycobactin in tissues heavily infected with *M. avium* SDZOO, *M. paratuberculosis* and *M. tubercu-*

losis, although mycobactin was detected in tissues seeded from primary cultures of *M. avium* SDZOO and other mycobactin-competent mycobacteria. This finding suggests that mycobacteria do not synthesize mycobactin *in vivo*, or at least, not at the same level that they do *in vitro*. Mycobactin may, therefore, be more important for mycobacterial growth outside of the host macrophage. A high iron concentration coupled with low pH or, alternatively, an iron-acquisition mechanism involving exochelins or some other yet-discovered siderophore would serve as an explanation for mycobacterial growth *in vivo*. Since we have been unsuccessful in our attempts to isolate or demonstrate exochelins or other siderophores, it seems more likely that conditions within the macrophage allow for iron acquisition by perhaps a non-siderophore-mediated mechanism.

The *in vivo* environment has been thought to be highly restrictive in iron availability. Although iron is more soluble at lower pH, the solubility of free iron at sites within the host where the pH is low, is reported to be only 10^{-12} which is not sufficient for growth of bacteria.[22] There is little information regarding the role of host iron-binding proteins in mycobacterial infections. Most iron in the host is found intracellularly bound to ferritin, transferrin, heme proteins and lactoferrin in phagocytic granules. The extracellular iron found in body fluids is bound to transferrin in serum and lymph and lactoferrin in milk and external secretions. It has been suggested that mycobactin can remove iron from transferrin and ferritin for bacterial utilization even though the affinity constants for mycobactin and transferrin, 10^{30} and $10,^{36}$ respectively, for iron, might suggest otherwise.[23,24] Binding of iron by transferrin and lactoferrin has been shown to vary with pH and is maximal above pH 7.0.[25] Our data confirms the dissociation of iron at lower pH from these proteins in CDM. As the pH is reduced to 6.5, iron begins to dissociate from transferrin and is completely dissociated at pH 4.5 whereas, lactoferrin retains its iron below pH 4. Although the nature of iron-binding sites in transferrin and lactoferrin are very similar, the two proteins differ in their amino acid, peptide and carbohydrate composition. Serum transferrin is normally found in concentrations of 20–30 μM and is 25–40% iron-saturated.

The iron content within macrophages is not known, but recent evidence suggests that, for intracellular pathogens, iron availability is not as limited as once thought.[18] As shown by color indicators[26] and pH-dependent probes[27] the pH of phagocytic vacuoles lies somewhere between 4.5–6.0. Studies have confirmed that, unlike most bacteria, the optimal pH for growth of *M. paratuberculosis* is approximately 5.5–6.0.[19,28] It is at this lower pH that growth of *M. paratuberculosis* can occur in the absence of mycobactin when sufficient iron is available. Thus, the lower pH environment within the macrophage may sustain growth of *M. paratuberculosis*.

Our results reaffirm that *M. paratuberculosis* is not capable of utilizing siderophores produced by unrelated organisms and that pathogenic mycobacteria do not synthesize mycobactin *in vivo* to their full capacity if at all, advancing the belief that iron acquisition within the host is not dependent on siderophore-mediated events. We suggest that the microenvironment in which mycobacteria are found within the host may not be a site of iron-deprivation and that growth *in vivo* may depend on host iron-binding proteins as iron sources. The observation that iron dissociates from transferrin and lactoferrin at low pH is not novel, but, taken together with the finding that *M. paratuberculosis* grows in the absence of mycobactin at low pH, might help describe a model of iron acquisition for mycobacteria and other intracellular bacteria within the host. The importance of understanding iron acquisition mechanisms is evident in a recent report describing the inhibition of intracellular growth of *Legionella pneumophila* by down-regulating transferrin receptors on activated macrophages with interferon-τ.[29]

Materials and methods

Bacterial strains. Strains of *M. paratuberculosis* included the type strain, *M. paratuberculosis* ATCC 19698, and a clinical strain, *M. paratuberculosis* UWIS B025, isolated from the ileum of a Guernsey cow with severe clinical signs of Johne's disease. *M. avium* SDZOO was recovered from the liver and spleen of a flamingo at the San Diego Zoo, CA. Clinical isolates were passaged only twice on chemically defined media prior to being maintained at −70°C and identification was supported by microbiological and histological findings. *M. phlei* and *M. smegmatis* were obtained from L. Kubista (Wisconsin State Laboratory of Hygiene, WI).

Media. A previously described iron-deficient chemically defined medium (CDM) was used to maintain organisms in an iron-deficient state.[19] Middlebrook 7H12B radiometric broth was used in all bioassay experiments that evaluated the ability of iron-binding compounds and tissue extracts to promote growth of *M. paratuberculosis* in the absence of exogenously added mycobactin. The effect of pH on growth of *M. paratuberculosis* in the bioassay was studied by adjusting the pH of 7H12B medium to values between 3.5–6.8 through the addition of 0.1 N HCL which did not significantly alter the volume. The pH of each vial was checked before and after growth experiments.

M. paratuberculosis *bioassay.* The ability of *M. paratuberculosis* to grow in mycobactin-free medium supplemented with iron-containing compounds or extracts of tissues was determined by a radiometric bioassay incorporating *M. paratuberculosis* ATCC 19698 and *M. paratuberculosis* UWIS B025. Both strains were conditioned so as to be mycobactin-free by cultivating the organisms for a minimum of 1 week in CDM in the absence of iron and mycobactin. The single-celled strains of *M. paratuberculosis* were inoculated (0.1 ml) into vials containing radiometric media using a Tridak Stepper™ repetitive syringe (Vangard International, Neptune, NJ). The inoculum size for *M. paratuberculosis* ATCC 19698 and *M. paratuberculosis* UWIS B025 was $10^{5.5}$ cfu and 10^7 cfu, respectively. Inoculated vials were gently swirled and incubated at 37°C without shaking. All supplements were added using the precision syringe device and tested with and without 1.2 μM mycobactin in the medium. Positive control vials contained *M. paratuberculosis* and 1.2 μM mycobactin while negative control vials contained only *M. paratuberculosis*. The extreme sensitivity of the bioassay allowed for detection of as little as 6 nM mycobactin.

Growth measurement. Growth of mycobacteria in ^{14}C-palmitate medium produced respired $^{14}CO_2$ which was detected by an automated ionization detector, BACTEC® (Johnston Laboratories, Cockeysville, MD). Growth measurements of individual vials were taken at multiple times during the 3 week incubation period. One growth unit is equal to 0.25 nCi of ^{14}C. Previous studies have demonstrated that the rate of $^{14}CO_2$ production is directly correlated to the number of viable organisms during logarithmic growth.[20] Growth rates were determined by plotting the log of growth units against time and graphically identifying the linear portion representing exponential growth. The slope was determined from the regression of this line and reported as growth rate. Graphically displaying growth over a period of time in terms of growth rate proved to be more instructive than showing entire growth curves.

Extraction of tissues for mycobactin-like activity. Tissues seeded with *M. phlei, M. smegmatis* or *M. avium* SDZOO were extracted for mycobactin with chloroform. Each of these mycobactin-producing strains were cultivated on low-iron CDM agar to induce synthesis of mycobactin. After 7 days incubation at 37°C for *M. phlei* and *M. smegmatis*, and 2 weeks for *M. avium* SDZOO, the cells were harvested from each plate and suspended in 5 ml sterile saline. The suspensions were then added to approximately 25 g of sterile homogenized bovine liver or normal colon contents and mixed well. After several hours, the tissues were extracted with 20 ml of chloroform and the extracts evaporated to dryness. The residue was taken up in a small volume of ethanol, filtered (0.2 μM-pore filter) and tested for the ability to promote growth of *M. paratuberculosis* in the bioassay described above. Unseeded tissue extracts served as negative controls.

Tissues, naturally infected with pathogenic mycobacteria, were examined for mycobactin in the same manner as the seeded tissues. Bovine intestinal tissues and colon contents were obtained from a cow with severe clinical signs of Johne's disease which was confirmed by pathological and microbiological findings. The ileum contained at least 10^7 cfu *M.*

paratuberculosis/g, whereas, colon contents comprised at least 10^8 cfu *M. paratuberculosis*/g. Liver and spleen containing approximately 10^8–10^9 cfu *M. avium*/g tissue were obtained at necropsy from a flamingo at the San Diego Zoo, CA. Pooled spleen homogenates from mice experimentally infected with *M. tuberculosis* ($10^{4-5.2}$/g) were kindly provided by D. W. Smith (Dept Medical Microbiology, University of Wisconsin-Madison, WI). Intact tissues were first homogenized using a Stomacher Lab-Blender 80 (Tekmar Co., Cincinnati, OH), extracted and processed as described above for the seeded tissues.

Iron-containing compounds. Bovine transferrin (Sigma Chemical Co., St Louis, MO), lactoferrin derived from bovine milk (US Biochemical Corp., Cleveland, OH) and apoferritin from horse spleen (US Biochemical Corp.) were all purchased in purified forms substantially iron-free. Transferrin and lactoferrin were reconstituted in buffer (reconstitution buffer) consisting of 10 mM HEPES-150 mM NaCl–10 mM sodium bicarbonate (pH 7.2). The proteins were saturated at various levels with iron by combining calculated amounts of ferric ammonium citrate dissolved in 0.1 M sodium citrate–0.1 M $NaHCO_3$ buffer (pH 7.0) with 2 mM of the respective protein at 4°C. The iron-bound proteins were then dialyzed against reconstitution buffer followed by iron-free water using Spectra/por™ 2 dialysis membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA). The protein solutions were filter-sterilized and the amount of bound iron and protein were confirmed using the ferrozine iron assay[21] and the protein assay of Bradford (Bio-rad, Richmond, CA), respectively. For iron-binding proteins, vials containing 7H12B medium were deferrated by making each culture vial 0.2 mM with respect to the iron chelator deferoxamine mesylate (Desferal®, Ciba Pharmaceutical Co., NJ). Iron-binding proteins were saturated between 25–100% with iron and assayed for growth-stimulating activity in final concentrations ranging from 10 μM to 100 μM. Hemin and hemoglobin (Sigma Chemical Co) were purchased in iron-saturated forms.

Ferrichrome and ferrichrome A were kindly provided by S. A. Leong (Dept Plant Pathology, University of Wisconsin-Madison, WI). Enterobactin and aerobactin were donated by J. B. Neilands (Dept Biochemistry, University of California-Berkeley, CA). Multocidin was a gift from S. W. Mahesharwan (Dept of Microbiology, University of Minnesota, MN). Deferrioxamine mesylate (Desferal®, Ciba Pharmaceutical Co., Summit, NJ) and Rhodotorulic acid (Sigma Chemical Co., St Louis, MO) were purchased.

Dissociation of iron from iron-binding proteins. The relative amount of iron which dissociated from iron-binding proteins with decreasing pH was determined with aliquots of CDM adjusted to various pHs between 3.6 and 7.8 after making it 1 mM with respect to 50% iron-saturated lactoferrin or transferrin, or 0.1 mM 100% iron-saturated ferritin. Each of the proteins demonstrated an absorbance peak at 450 nM when bound to iron and no absorbance in the iron-free form. Absorbance was measured spectrophotometrically at 450 nM.

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