Lack of a Role for Iron in the Lyme Disease Pathogen

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A fundamental tenet of microbial pathogenesis is that bacterial pathogens must overcome host iron limitation to establish a successful infection. Surprisingly, the Lyme disease pathogen *Borrelia burgdorferi* has bypassed this host defense by eliminating the need for iron. *B. burgdorferi* grew normally and did not alter gene expression in the presence of iron chelators. Furthermore, typical bacterial iron-containing proteins were not detected in cell lysates, nor were the genes encoding such proteins identified in the genome sequence. The intracellular concentration of iron in *B. burgdorferi* was estimated to be less than 10 atoms per cell, well below a physiologically relevant concentration.

Successful colonization of the human host by bacterial pathogens requires that bacteria overcome strict iron (Fe) limitations imparted by the host (*1*, *2*). In humans, the amount of free Fe ($\sim 10^{-18}$ M) (*3*) is well below the levels required to support the growth of most bacteria (10^{-6} to 10^{-7} M) (*4*). At the onset of infection, host cells increase the production and secretion of lactoferrin to limit further available Fe and inhibit bacterial growth (*5*). To overcome this Fe restriction, pathogenic bacteria have developed specialized systems that aid in the acquisition and assimilation of Fe. However, this does not seem to be the case for *Borrelia burgdorferi*.

Analysis of membranes of *B. burgdorferi* indicated that they lack metalloproteins commonly associated with bacterial cytoplasmic membranes (Table 1) (*6*). No cytochromes, respiratory proteins, or tricarboxylic acid metalloenzymes (e.g., succinate dehydrogenase) were detected in purified inner membranes. Analysis of the complete genome sequence confirmed that *B. burgdorferi* does not contain genes encoding these proteins, and in fact, contains very few genes encoding metalloproteins (*7*). The genes encoding a superoxide dismutase (*sodA*) (*8*), a putative ferric-uptake regulatory protein (*fur*), and a putative neutrophil-activating protein (*napA*) have been identified (*7*). Typically, these types of proteins require Fe as a cofactor, but their metal requirements have not been determined in *B. burgdorferi*. For example, the *B. burgdorferi* SodA has >50% identity with Mn-dependent enzymes from *Thermus thermophilus*, *Thermus aquaticus*, and *Bordetella pertussis* (*9*), and cambialistic enzymes (those active with either Fe or Mn as a cofactor) from *Porphyromonas gingivalis* (*10*) and *Streptococcus mutans* (*11*). Likewise, the Fur homolog of *B. burgdorferi* has 54.3% similarity to the *Bacillus subtilis perR* gene product, which requires Mn as a cofactor and regulates *dps*, *hemA*, *katA*, and *mrgA*, all of which are involved in responses to oxidative stress and metal limitation (*12*, *13*). Therefore, these *B. burgdorferi* proteins may be Mn-dependent rather than Fe-dependent.

To investigate these observations further, we examined the effect of Fe limitation on the growth of *B. burgdorferi*. Because *B. burgdorferi* is routinely grown on complex medium [Barbour-Stoenner-Kelly (BSK-II)] supplemented with 5 to 10% rabbit serum (*14*), it is difficult to manipulate the Fe concentration with chelators, such as 2,2′-dipyridyl (Dp) or deferoxamine mesylate (Desferal, Ds). Culturing *B. burgdorferi* on a modified serum-free (SF) medium supplemented with Excyte (SF-E) (*15*) resulted in growth rates and motility similar to those of cells grown in BSK-II. In contrast, cells cultured in SF medium were nonmotile and failed to grow (Fig. 1A). Comparisons of ^{35}S-labeled proteins isolated from cells grown in BSK-II or SF-E medium and analyzed by two-dimensional nonequilibrium pH gradient electrophoresis (2D-NEPHGE) indicated that protein profiles were not altered by growth in the modified medium. The SF-E medium, which contained 1 μM of Fe as determined by inductively coupled plasma-emission mass spectroscopy (ICP-MS), was free of siderophilins (e.g., transferrin) so that extracellular Fe concentrations could be manipulated experimentally with chelators.

B. burgdorferi cells were grown in SF-E medium or SF-E medium that contained 100 μM Dp or Ds. Reduced extracellular Fe concentrations in SF-E medium with chelators had no effect on the growth rate of *B. burgdorferi* (Fig. 1B), unlike *Escherichia coli*, which ceases to grow when extracellular Fe concentrations drop below 0.4 μM (*16*). This result is similar to the observation that the growth of *Lactobacillus plantarum*, a free-living soil bacterium that does not use Fe (*16*), is the same in Fe-chelated (10 μM ethylenediamine-*N*,*N*′-diacetic acid) and Fe-containing (0.6 μM) medium. *B. burgdorferi* cells cultured in SF-E medium treated with the metal-chelating resin Chelex (SF-E-Clx) were nonmotile and failed to grow (Fig. 1C). ICP-MS analysis of this medium indicated that Fe, Mn, and Zn were not detectable, and Mg concentrations had been reduced 100-

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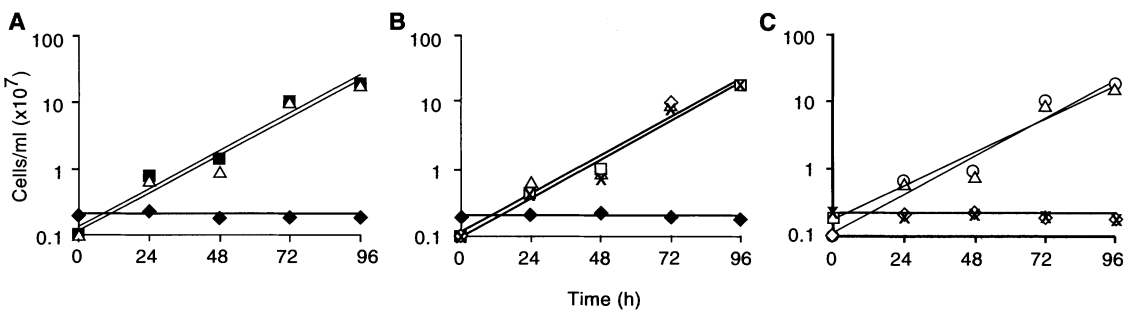

Fig. 1. *B. burgdorferi* cells are able to grow normally in Fe-limited medium. (**A**) Comparison of growth rates of cells grown in BSK-II (■), BSK-II without serum (SF medium) (◆), or SF medium supplemented with Excyte (SF-E) (△). (**B**) Comparison of growth rates of cells grown in BSK-II without serum (SF medium) (◆), SF-E medium (△), SF-E medium + 2-2′ dipyridyl (Dp) (□), and SF-E medium + Desferal (Ds) (×). (**C**) Comparison of growth rates of cells grown in SF-E medium △), SF-E medium treated with Chelex (SF-E-Clx) (◆), SF-E-Clx medium supplemented with Fe (×) or with Mg, Mn, and Zn (○). *B. burgdorferi* cells were grown in SF-E medium to a density of 5×10^7 cells per milliliter. A 5-ml culture was harvested and washed twice with 10 ml of buffer containing 20 mM Hepes, 100 mM NaCl, 10 mM EDTA (pH 7.6 with Hepes buffer), and cells were suspended in 5 ml of BSKII, SF, SF-E, SF-E + 100 μM Dp (or Ds), or SF-E-Clx. These reagents were used to inoculate 45 ml of equivalent media. $FeCl_3$, $MgCl_2$, $ZnCl_2$, and/or $MnCl_2$ were added to yield a final concentration of 10 μM, and cells were incubated at 34°C. Cell numbers were determined at 24-hour intervals by dark-field microscopy. Concentrations of metals in the different media were measured by ICP-MS (*17*).

fold (~60 μM). Various metals were added to the medium in order to restore growth. Mn (1 μM), Zn (1 μM), and Mg (600 μM) were all required to restore growth and motility, whereas the addition of Fe (1 μM) plus Mg (600 μM) failed to do so (Fig. 1C). Thus Mn, Zn, and Mg, but not Fe, are required for growth of *B. burgdorferi*.

Pathogenic bacteria respond to Fe limitation by coordinately regulating the expression of Fe-uptake systems and key virulence factors. Given that *B. burgdorferi* has little or no Fe requirement, we predicted that protein expression would not be altered under Fe-limited conditions in this pathogen. To test this hypothesis, we compared proteins isolated from cells grown in SF-E (1 μM Fe) or SF-E-Clx supplemented with Mg, Mn, and Zn (<0.1 μM Fe) medium by 2D-NEPHGE. No reproducible differences in protein profiles were observed. Likewise, the addition of ferrous sulfate, ferric chloride, or ferrous ammonium citrate (10 μM) to SF-E-Clx had no effect on protein patterns. Indeed, Fe concentrations >10 μM inhibited growth as well as motility and cell elongation.

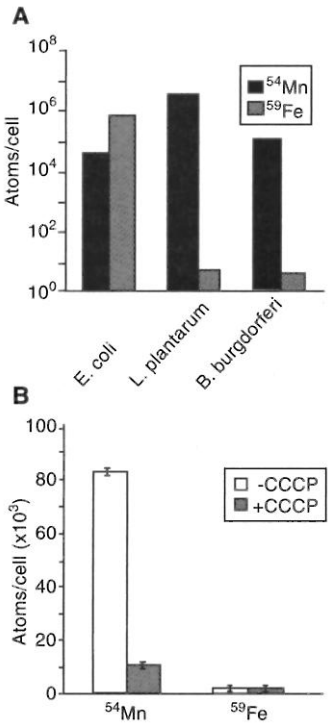

Fig. 2. *B. burgdorferi* accumulated ^{54}Mn, but not ^{59}Fe, in an energy-dependent manner. (**A**) *E. coli* strain MC4100 and *B. burgdorferi* strain B31cells were cultured in SF-E media to a density of 1 × 10^8 cells per milliliter. *E. coli* cells were incubated at 37°C with aeration for 12 hours, whereas *B. burgdorferi* cells were incubated at 34°C for 48 to 72 hours. *L. plantarum* cells were cultured in *Lactobacilli* MRS broth at 37°C to a density of 1 × 10^7 cells per milliliter. *E. coli* and *L. plantarum* cell numbers were determined by plate count, whereas *B. burgdorferi* cell numbers were determined as described above. Cells in each culture were harvested by centrifugation, washed twice in 10 ml of Hepes buffer, and suspended in SF-E media containing either ^{59}Fe or ^{54}Mn (0.16 μCi/ml each) at a concentration of 1 × 10^7 cells per milliliter. For determination of ^{54}Mn and ^{59}Fe uptake, the labeled cells were harvested, washed five times in 50 ml of Hepes buffer, and suspended in 500 μl of Hepes buffer. Incorporated radioactivity was determined from 10-, 50-, and 100-μl aliquots in 10 ml of scintillation fluid with a LS 6000 Beckman scintillation counter. (**B**) *B. burgdorferi* strain B31 cells were cultured and suspended in SF-E medium as described above. CCCP (100 μM) was added and cells were incubated at 34°C for 1 hour before adding either ^{59}Fe or ^{54}Mn. The cultures were incubated at 34°C for 12 hours. Uptake was determined as described above.

To determine the specific metal requirements of *B. burgdorferi* directly, we measured intracellular metal concentrations using ICP-MS (*17*). Intracellular levels of Fe, Mn, Ca, and Mg were compared with those in *E. coli* and *Treponema denticola*, two bacteria that require Fe, and in *L. plantarum*, which does not require Fe (Table 2). As expected, all bacteria tested contained high levels of Mg and Ca, while Mn levels in *L. plantarum* were consistent with those reported previously (*18*). Fe concentrations in cell lysates from *E. coli* and *T. denticola* were comparable, whereas Fe was below the detection limit in lysates of *L. plantarum* and *B. burgdorferi*. On the basis of ICP-MS, the intracellular concentration of Fe in *B. burgdorferi* was estimated to be <50 pmol per milligram of protein, or <10^2 to 10^3 atoms of Fe per cell. The intracellular levels of Mn were higher in *B. burgdorferi* than in the lysates of *E. coli* and *T. denticola*. Thus, like *L. plantarum*, *B. burgdorferi* accumulated Mn but not Fe to significant intracellular levels.

Table 1. *B. burgdorferi* does not contain metalloproteins commonly found in bacteria. The activity of the enzyme was assayed for in *B. burgdorferi* strain B31 cell extracts, and the presence or absence of the enzyme was confirmed from the genome sequence. ND, not determined.

Metalloprotein	Activity or protein detected	Gene present
Cytochrome	–	–
Cytochrome oxidase	–	–
Hydrogenase	ND	–
Catalase	–	–
Superoxide dismutase	+	+
Aconitase	–	–
Citrate synthase	–	–
Succinate dehydrogenase	–	–
Aldolase	ND	–
Glutamate synthase	ND	–
Glutamine synthetase	ND	–
Urease	ND	–
Ribonucleotide reductase	ND	–
RNA polymerase	ND	+
DNA polymerase	ND	+
Fur homolog	+	+

Uptake of ^{59}Fe and ^{54}Mn provided a more sensitive assay for determining intracellular metal concentrations (Fig. 2A). *E. coli* accumulated 7.1 × 10^5 atoms per cell of ^{59}Fe and 3.8 × 10^4 atoms per cell of ^{54}Mn, whereas *L. plantarum* contained 3.5 × 10^6 atoms per cell of ^{54}Mn and ^{59}Fe did not accumulate above background levels. Like *L. plantarum*, *B. burgdorferi* contained 8.3 × 10^4 atoms per cell of ^{54}Mn, but ^{59}Fe was undetectable. The specific activity of the ^{59}Fe used in these experiments should allow the detection of 10^2 to 10^3 atoms of ^{59}Fe per cell. When the specific activity of the ^{59}Fe was increased (0.5 μCi/ml) so that 75% of the total Fe was in the radioactive form, ^{59}Fe uptake in *B. burgdorferi* remained undetectable. This indicated that intracellular Fe levels were <10 atoms per cell, well below a physiologically relevant quantity. The ^{54}Mn uptake experiments suggested a requirement for Mn by *B. burgdorferi*. Currently, little is known about transport of essential nutrients in *B. burgdorferi* or other spirochetes. To determine if the transport of ^{54}Mn was energy-dependent, we used the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in uptake assays. CCCP inhibited ^{54}Mn accumulation by >75% (Fig. 2B), but had no effect on the background levels of ^{59}Fe. The uptake of ^{54}Mn by *B. burgdorferi* cells was linear for 1 hour.

We have shown that *B. burgdorferi* is unique among pathogenic bacteria in having evolved a novel strategy to survive Fe limitation in the human host. This has been accomplished by eliminating most of the genes that encode proteins that require Fe as a cofactor and, similar to *L. plantarum*, by substituting Mn for Fe in the few metalloproteins found in *B. burgdorferi*. *B. burgdorferi* is an obligate parasite with a minimal genome

Table 2. *B. burgdorferi* has extremely low levels of intracellular Fe as measured by ICP-MS. *E. coli* strain MC4100 and *B. burgdorferi* strain B31 cells were grown in BSK-II or SF-E medium, whereas *L. plantarum* 14917 was cultured in APT medium. Cells were harvested and washed five times in 10 ml of Hepes buffer. *E. coli* and *B. burgdorferi* cells were lysed by freeze-thaw in the presence of 2% SDS. *L. plantarum* cells were disrupted by three passages through a French pressure cell (1.1 × 10^5 kPa). One milliliter of each extract was assayed for metals as described (*17*). Protein concentrations were determined as described by Markwell *et al.* (*21*). The data represent an average of three samples from two independent experiments.

Bacterium	Metal*			
	Mg	Ca	Mn	Fe
E. coli	146	46	0.79	4.2
T. denticola	71	35	0.24	3.5
L. plantarum	120	4.5	150	< 0.05
B. burgdorferi	94	43	1.9	< 0.05

*Expressed as nanomoles of metal per milligram of protein

that lacks genes encoding the enzymes for most biosynthetic pathways. A consequence of this evolution to obligate parasitism has been the elimination of the requirement for Fe. This adaptation by *B. burgdorferi* has been successful, and other pathogenic bacteria with limited genomes, such as *T. pallidum* (950 Mb) (*19*) and *Mycoplasma pneumoniae* (650 kb) (*20*), may have adopted similar approaches to avoid host Fe limitation.

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15. SF-E medium consisted of serum-free BSK-II (*16*) with the following modifications. The *N*-acetylglucosamine and pyruvate were increased to 0.64 and 1.28 g/liter, respectively. The bovine serum albumin was increased from 4 to 7% (w/v), and the medium was supplemented with 3-phosphoglyceric acid (0.48 g/liter), 0.8% (v/v) glycerol, and 2% (v/v) Excyte.
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22. We thank T. Hoover and D. Krause for critical reading of the manuscript. Supported by National Institute of Allergy and Infectious Diseases grants AI33501 and AI144911.

1 February 2000; accepted 7 April 2000

Intracellular Parasitism by the Human Granulocytic Ehrlichiosis Bacterium Through the P-Selectin Ligand, PSGL-1

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Human granulocytic ehrlichiosis (HGE) is a febrile tick-borne illness caused by a recently discovered intracellular bacterium remarkable for its tropism for professionally phagocytic neutrophils. Monoclonal antibodies against the P-selectin binding domain of the leukocyte P-selectin glycoprotein ligand, PSGL-1, prevented HGE cell binding and infection, as did enzymatic digestion of PSGL-1. Furthermore, simultaneous neoexpression in nonsusceptible cells of complementary DNAs for both PSGL-1 and its modifying α-(1,3) fucosyltransferase, Fuc-TVII, allowed binding and infection by HGE. Thus, the HGE bacterium specifically bound to fucosylated leukocyte PSGL-1. Selectin mimicry is likely central to the organism's unique ability to target and infect neutrophils.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne infection that causes an acute febrile illness, which is often severe, and frequently coinfects individuals with Lyme disease (*Borrelia burgdorferi*), also carried by *Ixodes* ticks (*1*, *2*). In the few years since its recognition, the disease has been reported in several hundred individuals, primarily in the regions of the north central and northeastern United States, where Lyme disease is endemic, and seropositivity has been reported in several other countries. The etiologic agent is an obligate intracellular bacterium found in the peripheral blood granulocytes of infected patients and is closely related, or identical, to *Ehrlichia equi* and *E. phagocytophila* (*1*, *3*), which infect horses and ruminants, respectively. Several pathogens of humans have life cycles that involve permissive infection of monocytes, but the HGE agent is noteworthy in its tropism for and growth within professionally phagocytic granulocytes. The as of yet unnamed etiologic organism of HGE was recently isolated with the promyelocytic leukemia cell line, HL60, and it has since been shown that the organism can replicate in other myeloid cell lines and in normal human bone marrow progenitor cells (*3*–*5*). The tropism of this organism for leukocytes and its ability to evade normal phagocytic pathways and lysosomal fusion (*6*) suggest that the organism uses specific receptor-mediated pathway(s) for cellular adhesion and entry.

Although intracellular infection by bacteria is an area of intense investigation, the entry of pathogens into permissive pathways in normally hostile phagocytic cells remains poorly understood (*7*). Some organisms infect a variety of cells and bind to molecules that are widely distributed in the host (e.g., *Yersinia* species and *Toxoplasma gondii* to β-1 integrins) (*8*, *9*). Others are able to use specific leukocyte molecules in their adhesion to monocytes (e.g., *Legionella pneumophila*, *Leishmania donovanii*, and *Mycobacterium tuberculosis* through binding to complement receptors) (*10*, *11*). The receptors responsible for ehrlichial binding and entry into host cells were unknown.

Leukocyte cell surface expression of the sialyl Lewis x tetrasaccharide (sLe^x or CD15s) and the expression of the α-(1,3) fucosyltransferase, Fuc-TVII, responsible for its terminal fucosylation are linked to cell susceptibility to HGE infection (*12*). However, Fuc-TVII has multiple substrates, sLe^x itself modifies a wide variety of proteins and lipids, and antibodies to sLe^x (anti-sLe^x) did not block HGE adhesion to susceptible cells (*12*), suggesting that sLe^x alone is not a specific receptor for the organism. We hypothesized that the HGE receptor is a Fuc-TVII–modified cell surface glycoprotein expressed on neutrophils. The linkage of infection to the expression of sLe^x also suggested that the HGE receptor might be a selectin ligand.

We examined the effects of monoclonal antibodies (mAbs) against sLe^x itself and known sLe^x-modified or related mucin-modified proteins, including CD43, CD16, CD24, and the major identified selectin ligand of neutrophils (*13*, *14*), PSGL-1, on HGE binding (*15*). As shown by fluorescence-activated cell sorting (FACS) analysis (*16*) of binding of fluoresceinated bacteria, mAb PL1, which is directed against the NH_2-terminus of PSGL-1 (residues 8 to 21) and blocks P-selectin binding to cells (*17*), blocked the binding of HGE to HL60 cells in a dose-dependent manner (Fig. 1A). These results were confirmed with three different, geographically diverse strains of HGE. mAb KPL-1, which binds to the tyrosine sulfate motif of PSGL-1 and blocks binding to P-selectin (*18*), also blocked binding of bacteria to HL60 cells, whereas mAb PL2, which binds to a membrane proximal epitope on PSGL-1 and does not block binding to P-

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