Effects of iron modulation on growth and viability of *Rhodococcus equi* and expression of virulence-associated protein A

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**Objective**—To determine the importance of iron for in vitro growth of *Rhodococcus equi*, define potential iron sources in the environment and mechanisms by which *R equi* may obtain iron from the environment, and assess expression and immunogenicity of iron-regulated proteins.

**Sample Population**—10 virulent and 11 avirulent strains of *R equi*. 

**Procedure**—In vitro growth rates and protein patterns of *R equi* propagated in media with normal, excess, or limited amounts of available iron were compared. Immunoblot analyses that used serum from foals naturally infected with *R equi* and monoclonal antibody against virulence-associated protein (VapA) were conducted to determine immunogenicity and identity of expressed proteins.

**Results**—Excess iron did not alter growth of any *R equi* strains, whereas growth of all strains was significantly decreased in response to limited amounts of available iron. Virulent *R equi* were able to use iron from ferrated deferoxamine, bovine transferrin, and bovine lactoferrin. Only virulent *R equi* expressed an iron-regulated, immunogenic, surface-associated protein identified as VapA.

**Conclusions and Clinical Relevance**—Iron is required for the growth and survival of *R equi*. Sources of iron for *R equi*, and mechanisms by which *R equi* acquire iron in vivo, may represent important virulence factors and novel targets for the development of therapeutic and immunophylactic strategies to control *R equi* infection in foals. Expression of VapA is substantially upregulated when there is a limited amount of available iron. (Am J Vet Res 2003;64:1337–1346)

*Rhodococcus equi* is a gram-positive, soil-saprophytic, facultative-intracellular actinomycete bacterium that shares many microbiologic characteristics with members of the genera *Mycobacterium* and *Corynebacterium*.1,2 *Rhodococcus equi* proliferates in alveolar and other macrophages and causes severe and often fatal pneumonia in young foals and immunocompromised humans.3 Epidemiologic evidence indicates that foals that develop *R equi* infections are most commonly infected during the first few days after birth,4 whereas juvenile and adult horses are relatively refractory to infection.5,6 The onset of clinical signs of disease is most common in foals that are 30 to 60 days old but may not be apparent for several months.7,3

Virulence of *R equi* in foals is strongly associated with an 85- to 90-kilobase (kb) virulence plasmid.6,8 This plasmid encodes the production of 7 virulence-associated proteins (VAPs; ie, VapA, VapC, VapD, VapE, VapF, VapG, and VapH).5,7,9,10 The function of these proteins is unknown, and although they are associated with virulence, specific virulence factors for *R equi* have not yet been identified. Identification and characterization of *R equi* virulence factors and understanding of the mechanisms by which these bacteria cause disease are vitally important to disease prevention and control.

To survive in macrophages, *R equi* must be able to adapt to an intracellular environment that contains a powerful array of antimicrobial defenses. One of these defense mechanisms is the severe limitation of available (ie, free) iron.11,12 Availability and acquisition of iron is crucial for survival and growth of most bacterial pathogens in which iron metabolism has been investigated.13 The virulence of some bacterial pathogens is markedly enhanced when there are increased amounts of available iron.14,15 Despite the abundance of iron in host body fluids, the concentration of free iron is low (approx 10⁻¹⁰ M), primarily as a result of the iron-binding proteins (ie, transferrin, lactoferrin, and ferritin).11,16,17 Pathogenic bacteria frequently use the low-iron environment of the host as a signal to induce or repress various virulence factors.18 These virulence factors include high-affinity iron chelators, such as siderophores and membrane-associated iron-binding proteins.19,20 Iron-regulated siderophores and proteins, as well as some iron-regulated toxins such as the diphtheria toxin of *Corynebacterium diphteriae*, are often associated with mobile genetic elements.21 Diphtheria toxin, a virulence factor that interferes with protein synthesis, is encoded by a bacteriophage that
lyses C diphtheriae. The toxin gene is regulated by an iron-dependent diphtheria toxin repressor (DtxR) protein, and the toxin is only expressed in the absence of iron. Rhodococcus equi possesses an iron-dependent repressor gene (ie, IdeR) that encodes a protein that shares 57 and 75% homology with the DtxR proteins of C diphtheriae and Mycobacterium tuberculosis, respectively, with the highest degree of similarity in the N-terminal DNA-binding domains. Although the R equi IdeR gene has been expressed in Escherichia coli, expression of this protein by R equi has not been documented, and its function has not been determined. To the authors’ knowledge, investigations of the importance of iron for the survival of R equi and the mechanisms by which R equi acquire and use iron have not been reported, except for 1 report that indicated R equi does not produce mycobactin, a siderophore that is specific for Mycobacterium and Nocardia, organisms that are closely related to R equi.

We hypothesized that iron was crucial for survival of R equi and that mechanisms of iron acquisition are important for virulence. To address these hypotheses, we investigated the in vitro growth and protein expression of R equi in response to variations in iron availability. The purposes of the study reported here were to develop a protocol for reducing iron in vitro to mimic the in vivo availability of iron to R equi, evaluate the effects of altered iron availability on the capacity of virulent and avirulent R equi to proliferate, assess the ability of various iron chelators to modulate growth of R equi, and determine whether R equi expresses proteins in response to excess or limited amounts of iron.

Materials and Methods

Bacteria—Three groups of R equi (10 virulent strains [vapA-positive strains], 10 avirulent strains [vapA-negative strains], and 1 isogenic strain from which the virulence plasmid was cured) were used in the study. All 21 strains of R equi had been isolated from foals (strains American Tissue Culture Collection [ATCC] 33701P+, 97-7, 97-8, 97-9, 97-10, 97-12, 97-16, and 97-22) and humans (strains H-8 and H-9). The vapA-positive strains were obtained from foals (strains American Tissue Culture Collection [ATCC] 33701P+, 97-7, 97-8, 97-9, 97-10, 97-12, 97-16, and 97-22) and humans (strains H-8 and H-9). The vapA-negative strains were obtained from soil (strains 16-N1, 16-P1, 11-N3, 22-N3, 39-P2, and 39-P3), humans (strains H-6 and H-7), and a foal (strain 97-4). The isogenic plumcid-cured strain (ATCC 33701P+) was generously provided by another researcher.

Growth conditions—Initially, bacteria were grown in brain-heart infusion broth supplemented with 10% newborn calf serum for 48 hours at 35°C while being rotated at 10 rounds/min. Bacteria were centrifuged at 1,600 × g for 10 minutes, washed 3 times with PBS solution, and inoculated into R equi minimal media. Minimal media consisted of 5 g of K2HPO4/L, 1.5 g of NaH2PO4 • H2O/L, 0.2 g of MgSO4 • 7 H2O/L, 1 g of (NH4)2SO4/L, 0.1 m Na thiamine, 20 mM lactate, and 0.2 mL of trace element solution. Trace element solution consisted of 50 g of EDTA/L, 22 g of ZnSO4 • 7 H2O/L, 5.34 g of CaCl2/L, 5.06 g of MnCl2 • 4 H2O/L, 4.99 g of FeCl3 • 7 H2O/L, 1.10 g of (NH4)3MoO4 • 4 H2O/L, 1.57 g of CuSO4 • 5 H2O/L, and 1.16 g of CoCl2 • 6 H2O/L, and it was adjusted to pH 6.0 by the addition of KOH. All media were prepared with sterile water. All reagents used in the minimal media were obtained from a single source, and all were reagent grade, except thiamine and lactate. Each final medium was adjusted to pH 7.0 by the addition of 1 N NaOH.

Minimal medium with iron (MM) was used as the control media in all experiments, and minimal medium without added iron (MM-Fe; iron-limited medium) was used in all experiments involving iron modulation. All media were prepared in polypropylene beakers, sterilized through polystyrene filters, and stored in the filter containers. Iron concentrations of the various media preparations were determined by use of inductively coupled plasma optical emissions spectrophotometry. Iron concentration in MM was 2.9 µM, whereas iron concentration in MM-Fe was 0.5 µM. Rhodococcus equi were added to the various media preparations at concentrations of 5 × 106 organisms/mL and incubated at 35°C for 48 hours while being rotated at 10 rounds/min, unless indicated otherwise.

Bacterial quantification—A growth curve for R equi cultured for 48 hours in MM was determined, as reported elsewhere, by use of plate counts of 10-fold serial dilutions cultured on trypticase soy agar with 5% sheep RBCs and optical density (OD) measured by use of a spectrophotometer. The spectrophotometer result was the OD determined at a wavelength of 600 nm (OD600). Bacterial concentrations for the growth curve were determined at 0, 8, 12, 14, 16, 20, 24, 36, and 48 hours after initiation of culture. From these data, the number of colony-forming units (CFUs) of R equi was correlated with OD600 values and programmed into the spectrophotometer, in accordance with the manufacturers directions, for automated calculation of CFU values. Plate counts of serial dilutions were used to quantify bacteria when concentrations were < 1.0 × 108 organisms/mL, and spectrophotometric analyses were used to calculate CFU concentrations when bacterial concentrations were ≥ 1.0 × 108 organisms/mL. All bacterial concentrations were reported as number of CFUs per milliliter. Bacterial growth was monitored in all experiments at 0, 8, 24, and 48 hours after initiation of culture unless otherwise stated, and all growth studies were conducted in duplicate.

Supplemental iron—Excessive iron concentrations were attained by supplementing MM-Fe with ferric chloride (FeCl3; 1,000 mg of Fe/kg in a 2% solution of hydrochloric acid). Initially, growth rates of 3 virulent strains of R equi (ATCC 33701P+, 97-8, and H-9) were compared when growth responses to excess iron cultured in MM and MM-Fe containing 25, 50, or 75 µM FeCl3, to assess growth responses to excess iron. Iron concentration in MM-Fe plus 25 µM FeCl3 was 24.8 µM, whereas iron concentrations in MM-Fe plus 50 µM FeCl3, and MM-Fe plus 75 µM FeCl3 were 50.2 and 74.6 µM, respectively. Subsequently, growth rates of 10 virulent and 11 avirulent strains of R equi cultured in MM and MM-Fe supplemented with 75 µM FeCl3 were compared.

Iron chelation—Mechanisms by which availability of iron could be limited were assessed by supplementing MM-Fe with 2 chelators (deferoxamine mesylate [DFO] and 2,2-dipyridyl [DIP]). The 2 iron chelators were used in separate experiments, because some bacteria can use the ferrated form of DFO. DFO was added to attain final concentrations of 500, 750, and 1,000 µM. Alternatively, DIP was added to attain final concentrations of 100, 150, and 200 µM. We were not able to measure free iron in media supplemented with chelators. Growth rates of 3 virulent strains of R equi (ATCC 33701P+, 97-8, and H-9) cultured in MM and MM-Fe supplemented with each of the various concentrations of DFO or DIP were compared. Growth rates of 10 virulent and 11 avirulent strains of R equi cultured in MM and MM-Fe supplemented with 200 µM DIP were also compared.
To confirm that iron was chelated by DIP and that growth inhibition was not attributable to chelation of other metals or toxic factors, iron-override studies were conducted by use of equimolar concentrations of FeCl₃. Growth rates of 3 virulent strains of R. equi (ATCC 33701P+, 97-8, and H-9) cultured in MM, MM-Fe plus 200µM DIP, and MM-Fe plus 200µM FeCl₃ were compared.

To determine whether R. equi could acquire and use iron bound to DFO in the presence of DIP, 3 virulent strains of R. equi (ATCC 33701P+, 97-8, and H-9) were grown in MM, MM-Fe plus 500µM DFO, MM-Fe plus 100µM DIP, or MM-Fe plus 500µM DFO plus 100µM DIP.

Iron-binding proteins—The capacity of mammalian iron-binding proteins to modulate growth of R. equi was determined by comparing growth rates of 3 virulent strains of R. equi (ATCC 33701P+, 97-8, and H-9) cultured in MM and MM-Fe supplemented with 30µM bovine holotransferrin (bHTf, 99% iron-saturated), 30µM bovine lactoferrin (bLf, 17% iron-saturated), or 30µM bovine apotransferrin (bATf, 0% iron-saturated). The ability of R. equi to acquire and use transferrin-bound iron was determined by comparing growth rates of 3 virulent strains of R. equi (ATCC 33701P+, 97-8, and H-9) cultured in MM, iron-limited media (ie, MM-Fe plus 100µM DIP), and iron-limited media supplemented with 50µM bHTf, 50µM bLf, or 50µM bATf.

Protein extraction—Proteins of R. equi were prepared as described elsewhere with slight modifications. Briefly, 3 virulent (ATCC 33701P+, 97-8, and H-9), 3 avirulent (ATCC 33703, 16-P1, and H-7), and 1 isogenic plasmid-cured mutant R. equi (ATCC 33701P–) were cultured in MM, iron-excess media (MM-Fe plus 75µM FeCl₃), and iron-limited media (MM-Fe plus 75µM DIP) for 120 hours at 35°C while being constantly rotated (10 rounds/min); cultures achieved a final concentration of 10⁹ bacteria/mL. For iron-limitation experiments, R. equi were cultured in MM-Fe plus 75µM DIP to suppress, but not completely inhibit, growth. The effect of iron modulation on protein expression was determined by comparing SDS-PAGE patterns of proteins from R. equi cultured in MM, MM-Fe plus 75µM FeCl₃, and MM-Fe plus 75µM DIP. Bacteria were washed 3 times with PBS solution, solubilized in Laemmli sample buffer (in accordance with the manufacturer’s protocol), and boiled for 10 minutes. Undissolved material was sedimented by centrifugation at 4,300 × g for 3 minutes. The supernatant contained proteins of approximately 10⁷ R. equi/10 µL of sample buffer.

Immune serum—Serum was obtained from six 6- to 12-week-old foals that were naturally infected with vapA-positive R. equi and from a newborn foal prior to ingestion of colostrum. Hyperimmune serum was obtained from a healthy mature Quarter Horse mare vaccinated with viable R. equi (ATCC 33701P–) and monovalent antibody. Serum and plasma were diluted 1:10,000 in 5% nonfat milk in TBST solution, and 17% iron-saturated). The ability of R. equi to acquire and use transferrin-bound iron was determined by comparing growth rates of 3 virulent strains of R. equi (ATCC 33701P+, 97-8, and H-9) cultured in MM, iron-limited media (ie, MM-Fe plus 100µM DIP), and iron-limited media supplemented with 50µM bHTf, 50µM bLf, or 50µM bATf.

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Protease treatment of R. equi—Location of expressed proteins was evaluated by semiquantitatively determining the relative concentrations of the 23-kd protein.

Electrophoresis and immunoblotting—We performed SDS-PAGE in accordance with the technique of Laemmli by use of mini-gels (15% Tris-glycine). Sample buffer (10 µL) containing the whole-cell antigens of approximately 10⁷ R. equi or 10 µL of stained broad-range SDS-PAGE markers was added to each lane and electrophoresed at 155 V for 1 hour. After completion of SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes for immunologic analysis. The PVDF membranes were incubated in 5% nonfat milk in Tris-buffered saline and 0.09% Tween 20 (TBST) solution for 60 minutes with gentle rock-
tured in MM and MM-Fe supplemented with 75µM FeCl₃. At 24 hours after initiation of culture, mean concentrations of virulent strains in control media and iron-supplemented media were 4.83 ± 0.25 × 10⁸ and 4.72 ± 0.52 × 10⁸ organisms/mL, respectively, whereas mean concentrations of avirulent strains in control media and iron-supplemented media were 4.98 ± 0.35 × 10⁸ and 4.83 ± 0.43 × 10⁸ organisms/mL, respectively. All strains grew at equivalent rates in MM and media with excess iron.

Effects of DFO on bacterial growth—The addition of DFO to culture media caused a significant ($P < 0.001$) increase in bacterial concentrations for all conditions over time. At 24 hours after initiation of culture, mean bacterial concentrations in MM and MM-Fe supplemented with 500, 750, or 1,000µM DFO increased from initial values of 5.00 × 10⁸ organisms/mL to 3.68 ± 0.19 × 10⁹, 3.16 ± 0.29 × 10⁹, 3.68 ± 0.19 × 10⁹, and 4.35 ± 0.35 × 10⁹ organisms/mL, respectively. There was not a significant difference in growth rates among bacteria cultured in MM and any of the media that contained DFO.

Effects of DIP on bacterial growth—Addition of DIP to culture media caused an exponential reduction in numbers of viable bacteria for all strains. At 48 hours, mean bacterial concentrations in media supplemented with 100, 150, or 200µM DIP had decreased from initial values of 5.00 × 10⁸ organisms/mL to 1.64 ± 0.91 × 10⁷, 7.25 ± 1.60 × 10⁶, and 2.03 ± 1.29 × 10⁶ organisms/mL, respectively. There was a 100-fold increase in bacterial CFUs for all 3 virulent strains of *R. equi* cultured in MM. The mean concentration increased from an initial value of 5.00 × 10⁸ organisms/mL to 6.92 ± 0.56 × 10⁸ organisms/mL at 48 hours after initiation of culture. There were significantly ($P < 0.001$) fewer bacteria in all media containing DIP than in MM without DIP. The decrease in number of *R. equi* media containing DIP appeared to be dose-dependent, with the greatest response for the highest DIP concentrations; however, there was not a significant difference in bacterial concentrations among media with various concentrations of DIP.

Table 1—Effect of excess iron on 2,2’-dipyridyl (DIP)-induced inhibition of the growth of *Rhodococcus equi*

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<thead>
<tr>
<th>Media</th>
<th>Culture time (h)</th>
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<td></td>
<td>0</td>
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<tr>
<td>MM</td>
<td>5.00 × 10⁸</td>
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<tr>
<td>MM-Fe plus 200µM DIP</td>
<td>5.00 × 10⁸</td>
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<tr>
<td>MM-Fe plus 200µM DIP plus 200µM FeCl₃</td>
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Values reported are mean ± SD cell density (number of colony-forming units [CFUs] per milliliter) for duplicate experiments for 3 virulent strains of *R. equi*. Cell densities between 1 × 10⁸ and 1 × 10⁹ cells/mL were determined spectrophotometrically, whereas densities < 1 × 10⁶ cells/mL were determined by enumerating the number of CFUs in serial dilutions plated on trypticase soy agar with sheep RBCs.

MM = Minimal media (contained 2.9µM Fe). MM-Fe = Minimal media without added iron (contained 0.5µM Fe). Within a column, values with different superscripts differ significantly ($P < 0.05$).

Table 2—Effects of DIP and deferoxamine mesylate (DFO) on growth of *R. equi*

<table>
<thead>
<tr>
<th>Media</th>
<th>Culture time (h)</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>MM</td>
<td>5.00 × 10⁸</td>
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<td>MM-Fe plus 500µM DFO</td>
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<td>MM-Fe plus 500µM DFO plus 100µM DIP</td>
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<td>MM-Fe plus 500µM DFO plus 100µM DIP</td>
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See Table 1 for key.
pared with the number of similar to that of bacteria cultured in MM. When com-

for bacteria cultured in iron-limited media supple-

P

number of CFUs was significantly (P < 0.001) lower

Use of DFO-bound iron—In an effort to deter-

mine whether the lack of inhibition of bacterial
growth by DFO was attributable to an inability to
sufficiently chelate iron or to the ability of R equi
to acquire iron bound to DFO, 3 virulent strains of R equi were cultured in MM, MM-Fe plus 500μM DFO, MM-Fe plus 100μM DIP, and MM-Fe plus 500μM DFO plus 100μM DIP. There was a significant (P < 0.001) increase in concentrations of R equi cultured in MM and in media containing 500μM DFO or 500μM DFO plus 100μM DIP, compared with concentrations of bacteria cultured in media that contained only 100μM DIP (Table 2).

Growth inhibition by iron-binding proteins—We detected a significant (P < 0.001) increase in the concentration of R equi cultured in media supplemented with 30μM bHTf and 30μM bLf, but there was not a significant increase in the concentration of bacteria cultured in media supplemented with 30μM bATf (Table 3). Bacterial growth was inhibited initially in media containing bHTf, but normal growth resumed by 24 hours. Growth of R equi in media containing bHTf or bLf was similar to growth of bacteria cultured in MM by 24 hours, whereas growth in media containing bATf was significantly (P < 0.001) less.

Use of iron bound to mammalian iron-binding proteins—The number of CFUs of R equi was significantly (P < 0.001) increased within 24 hours when cultured in iron limited media (MM-Fe plus 200μM DIP) supplemented with 50μM bHTf, and the number was similar to that of bacteria cultured in MM. When compared with the number of R equi grown in MM, the number of CFUs was significantly (P < 0.001) lower for bacteria cultured in iron-limited media supple-

Table 3—Effect of various iron-binding proteins on growth of R equi

<table>
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<th>Media</th>
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<td></td>
<td>0</td>
<td>8</td>
<td>24</td>
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<tr>
<td>MM</td>
<td>5.00 × 10^6</td>
<td>3.35 ± 2.91 × 10^6</td>
<td>4.54 ± 0.38 × 10^6</td>
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<tr>
<td>MM-Fe plus 30μM bHTf</td>
<td>5.00 × 10^6</td>
<td>3.31 ± 3.1 × 10^6</td>
<td>6.66 ± 0.97 × 10^6</td>
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<tr>
<td>MM-Fe plus 30μM bATf</td>
<td>5.00 × 10^6</td>
<td>8.07 ± 0.78 × 10^6</td>
<td>1.19 ± 0.26 × 10^6</td>
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bHTf = Bovine holotransferrin. bLf = Bovine lactoferrin. bATf = Bovine apotransferrin. See Table 1 for key.
mented with 50 µM bLf or 50 µM bATf. When compared with the number of R equi grown in MM and iron-limited media supplemented with bHTf, bLf, or bATf, the number of CFUs was significantly (P < 0.001) lower for bacteria cultured in iron-limited media (MM-Fe plus 200 µM DIP; Table 4).

Effect of iron modulation on protein expression—Protein patterns for virulent and avirulent R equi cultured in iron-replete and iron-limited media at 35°C were plotted (Fig 1). Virulent strains of R equi expressed a protein pattern consisting of a primary diffuse band with a molecular weight of approximately 23 kd, a 15- to 17-kd band, and, on a somewhat less consistent basis, a 44-kd band; all bands were highly immunogenic on immunoblot analysis with immune serum and upregulated in response to limited iron. Of the sera from 6 foals naturally infected with R equi, 2 revealed the protein pattern at a dilution of 1:2,000, 2 revealed it at a dilution of 1:500, and 2 revealed it at a dilution of 1:100. Serum from a horse immunized with virulent R equi, as well as commercially available R equi hyperimmune plasma, contained antibody that revealed the protein pattern at a dilution of 1:2,000. Precolostral foal serum (devoid of IgG) did not reveal any proteins. Expression of the 23-kd protein was significantly (P < 0.001) upregulated (approx 4-fold) in iron-limited media. There was not a significant difference in expression of the protein by virulent R equi cultured in MM (2.9 µM Fe) or media with excess iron (75 µM FeCl₃; data not shown). Avirulent strains of R equi did not express the 23-kd protein in any culture conditions.

Proteinase treatment of R equi—Treatment of whole virulent R equi (ATCC 33701P+) with trypsin and proteinase K abolished expression of the iron-regulated protein pattern (ie, 15- to 17-kd, 23-kd, and 44-kd bands; Fig 2). Immunoblot analysis—The VapA monoclonal antibody (ie, 10G5) revealed the iron-regulated protein pattern at a dilution of 1:10,000, as described elsewhere. Results for the VapA monoclonal antibody immunoblot had a pattern similar to that reported elsewhere (Fig 3)

Discussion
Iron is critically important for the growth of most pathogenic bacteria in which it has been investigated, and iron-regulated proteins are virulence factors for many bacteria. However, we are not aware of any published reports of the requirement, acquisition, or use of iron by R equi. In the study reported here, we established an in vitro protocol for limiting availability of iron to R equi, documented that iron is essential for survival of R equi, revealed that virulent and avirulent
strains of *R. equi* live or die at similar rates in media with excess iron or iron-limited media, determined that virulent *R. equi* can acquire iron from bHTF, bLf, and bATF, and determined that expression of VapA is substantially upregulated in response to limited amounts of iron.

The MM contained approximately 2.9 µM iron, and iron concentrations could readily be altered by addition of FeCl₃, or iron inhibitors; thus, it was used as the control growth medium. When no supplemental iron was added to the media (ie, MM-Fe), the iron concentration was approximately 0.5 µM. There was not a significant difference in the rate of growth of virulent *R. equi* cultured in MM or MM-Fe, which is similar to results reported elsewhere. Studies that used other bacteria suggest that iron concentrations < 0.1 µM are required to mimic in vivo iron availability and thereby induce bacterial iron stress.

On the basis that growth of *R. equi* was similar regardless of the addition of iron to the culture media, it was concluded that iron-chelating agents would likely be required to sufficiently limit the amount of available iron in MM-Fe. Initially, growth rates of 3 virulent strains of *R. equi* were assessed when cultured in MM-Fe supplemented with various concentrations of DIP. Bacterial concentrations decreased with increasing concentrations of DIP, and the maximum response was achieved with 200 µM DIP. To compare the effects of severe limitations of iron on growth of virulent and avirulent strains of *R. equi*, 10 virulent and 11 avirulent strains were cultured in media supplemented with 200 µM DIP. Growth of virulent and avirulent strains was severely and similarly inhibited, compared with growth of strains cultured in control media. In fact, many of the bacteria cultured in media containing DIP died, as indicated by marked reductions in the number of CFUs throughout the study.

Iron-override experiments were then performed to determine whether the suppressive and lethal effects of DIP were solely a result of iron chelation, or whether it was attributable to toxic effects or chelation of other metal ions. The addition of equimolar concentrations of FeCl₃ completely neutralized the growth-suppressive and bactericidal responses to DIP, and growth rates were similar to those of bacteria cultured in control media (Table 1). Therefore, it was concluded that DIP-induced growth suppression was attributable to chelation of iron and that iron was crucial for the survival and growth of *R. equi*.

Desferrioxamine, a siderophore produced by Streptomyces pilosus, is an effective iron chelator. A synthetic analogue of desferrioxamine (ie, DFO) is the chelator most commonly used in human patients to reduce iron concentrations in several conditions. This chelator was evaluated in our study because of its therapeutic potential for animals with *R. equi* infections. There was not a significant difference in the rate of growth of *R. equi* when cultured in control media or media containing various concentrations of DFO. In an effort to determine whether the lack of growth inhibition was a failure of the DFO to adequately chelate iron or to the ability of *R. equi* to access iron bound to DFO, *R. equi* was cultured in media that contained DIP and DFO. When DIP and DFO were combined, the growth-suppressive effects of DIP were blocked, and the rate of *R. equi* growth was the same as that of bacteria cultured in control media (Table 2). Analysis of these data strongly suggests that *R. equi* is capable of acquiring and using iron bound to DFO. Several other strains of bacteria (eg, *V. enterocolitica, Salmonella Typhimurium*, and *V. vulnificus*) can use iron bound to DFO to enhance growth and virulence. Although unlikely, it is possible that DFO may have interfered with the chelation of iron by DIP. On the basis of these data, DFO chelation should not be used for the treatment of animals with *R. equi* infections or the management of foals or humans at high risk of infection with *R. equi*.

We used FeSO₄ as the source of iron in the control media. Based on the fact that the addition of excessive amounts of FeSO₄ would result in the formation of precipitates, it was necessary to use FeCl₃ as the source of iron for the excess-iron media. To determine whether the difference in iron sources affected rates of growth, virulent *R. equi* were cultured in MM in which the source of iron was equimolar concentrations (ie, 2.9 µM) of FeSO₄ or FeCl₃; concentrations of *R. equi* increased exponentially over time in both media formulations, and there was not a significant difference in growth rates between media containing FeSO₄ or FeCl₃.

Excess iron enhances the growth and virulence of pathogens belonging to several genera of bacteria (eg, *Neisseria, Bacillus, Mycobacterium*, and *Staphylococcus*). To determine whether increased iron availability would enhance the growth of *R. equi*, 3 virulent strains were cultured in control media and in media with varying concentrations of excess amounts of iron. All treatments significantly promoted exponential growth of *R. equi* within 24 hours after initiation of culture, but there was not a significant difference in growth rates among bacteria cultured in media with the various iron concentrations, thereby indicating that excess amounts of iron do not enhance growth of *R. equi*.

Ten virulent and 11 avirulent strains of *R. equi* were cultured in media with severely limited iron and excessive iron to determine whether their growth responses would differ. Bacteria did not survive in iron-limited media, but there was not a significant difference between virulent and avirulent strains. Growth of virulent and avirulent strains in media with excessive iron was similar to that of bacteria cultured in control media, and there was not a significant difference between virulent and avirulent strains. On the basis of these findings, subsequent experiments on bacterial growth were conducted only for virulent strains. Although growth patterns of virulent and avirulent strains of *R. equi* were similar in response to severe limitations of iron, it is possible that they may have differed in response to more modest limitations in the amount of iron.

Transferrin and lactoferrin are mammalian proteins that function in a complementary manner to scavenge body tissues and bind nonprotein-bound free iron. In this manner, they exert antibacterial and
anti-inflammatory effects. \(^41\) It has been proposed that lactoferrin may be useful in the management of various bacterial infections because of its ability to chelate iron. \(^41,42\) Some bacteria, however, develop mechanisms by which they can assimilate and use transferrin- or lactoferrin-bound iron. \(^20\) Those mechanisms include synthesis and secretion of low-molecular-mass iron chelators (ie, siderophores) and direct contact between a surface receptor and the host transferrin. \(^31,32\) These mechanisms have been extensively studied \(^13,19,20\) in gram-negative organisms; however, the means by which gram-positive bacteria use transferrin-bound iron have not been fully elucidated.

On the basis of these competing strategies by the hosts and pathogens for limiting or acquiring iron, respectively, we evaluated the ability of bHTF, bLf, and bATf to modulate growth of \(R\) \(equi\). Bacterial growth was inhibited initially in media containing bLf, but normal growth resumed by 24 hours, suggesting that the system for use of lactoferrin was inducible or that another system released iron from the bLf. Bovine holotransferrin did not affect bacterial growth, whereas bATf inhibited growth (Table 3). In a separate study designed to determine whether \(R\) \(equi\) could acquire and use iron from bovine transferrins, the bacteria were propagated in iron-limited media with or without bHTF, bLf, or bATf. Bovine holotransferrin (99% iron-saturated) abolished the DIP-induced growth suppression response, whereas bLf (17% iron-saturated) and bATf (0% iron-saturated) moderated the response (Table 4). Collectively, these data suggest that bATf and bLf have the capacity to sequester iron and thereby suppress growth of \(R\) \(equi\); however, once saturation of these iron-binding proteins increases, bacteria have the ability to acquire and use that iron, and growth is restored. As reported in another study, \(^35\) the affinity of lactoferrin for iron is approximately 260-fold greater than the affinity of transferrin for iron. As transferrin saturation increases above 35%, its affinity is further decreased. \(^41\) On the basis of these characteristics, and because holotransferrin is more highly saturated with iron than are lactoferrin or apotransferrin, it seems logical that iron may be released more readily from holotransferrin. Although apotransferrin was not initially iron-bound, it may have bound iron in the media prior to chelation by DIP, thereby making it available to bacteria. Alternatively, the transferrins may interfere with chelation of iron by DIP. Whether \(R\) \(equi\) bind directly to these proteins to access iron or make use of siderophores to acquire iron remains to be determined.

Virulence of \(R\) \(equi\) in horses is strongly associated with an 85- to 90-kb plasmid that contains a putative 27,536-base pair (bp) pathogenicity island containing 7 virulence-associated genes (ie, \(vapA\), \(vapC\), \(vapD\), \(vapE\), \(vapF\), \(vapG\), and \(vapH\)). \(^6\) VapA is a surface-expressed protein \(^35\), VapC, VapD, and VapE are secreted \(^10\), and the expression characteristics of VapF, VapG, and VapH have not yet been described. Virulence-associated protein A, which has been most extensively studied, is associated with virulence. Although VapA without the remainder of the plasmid is not responsible for virulence \(^10\), \(^35\) results of a study involving deletion of the \(vapA\) gene and complementation provide compelling evidence for its role in virulence. The nature of that role, however, has not been defined. Of the 69 open-reading frames identified on the plasmid, functions have not been determined for 37, indicating that numerous other proteins may be encoded by the plasmid. \(^9\) Therefore, it is quite possible that other proteins encoded by the plasmid may be directly or indirectly responsible for virulence.

Experiments to determine differential expression, wherein \(R\) \(equi\) were cultured in iron-limited media to mimic conditions encountered in vivo, resulted in markedly increased expression of a protein pattern (ie, 15- to 17-kd, 23-kd, and 44-kd bands) by virulent strains (Fig 1). These proteins were produced in small amounts in bacteria cultured in control and excess-iron media but were significantly upregulated in response to iron-limited media. Avirulent strains of \(R\) \(equi\) did not express these proteins for any of the conditions tested. In addition, there was not a significant difference in expression of the proteins by virulent \(R\) \(equi\) cultured in control or excess-iron media. Our inability to detect iron-regulated antigens in whole \(R\) \(equi\) treated with trypsin and proteinase K suggested that they were exposed at the bacterial surface (Fig 2). Serum obtained from foals naturally infected with \(R\) \(equi\) and adult horses immunized with virulent \(R\) \(equi\) reacted strongly to the proteins during immunoblotting, indicating that the antigens were expressed in vivo and suggesting that they may be immunodominant antigens and important virulence factors.

On the basis of observed similarities in expression patterns between the proteins upregulated in response to iron limitations in the study reported here and results previously described for VapA, \(^35,37\) immunoblots with VapA monoclonal antibody and serum from a foal immunized with virulent \(R\) \(equi\) reacted strongly to the proteins in our study included a diffuse band at approximately 23 kd and a band at approximately 44 kd, similar to those described elsewhere. \(^35\) The 15- to 17-kd and 23-kd bands have been attributed to lipid modification of the VapA protein, and it has been suggested that the 44-kd band is a detergent-induced aggregation of proteins or a protein-detergent micelle. \(^37\)

A microarray analysis of virulence-plasmid gene induction by \(R\) \(equi\) cultured in equine macrophages for various environmental conditions revealed that vapA was induced (> 5-fold increase) in response to iron limitations. \(^17\) That increase in gene expression correlates well with the approximate 4-fold increase in protein expression observed in the study reported here. In the microarray study, limiting the amount of iron also resulted in an approximate 5-fold increase in the expression of vapC and more modest increases in expression of vapD, vapE, vapF, vapG, and vapH. \(^44\) In addition, the \(R\) \(equi\) genome contains a large number of genes involved in iron acquisition that are homologous with those in other pathogens. \(^45\) Results of those studies, in conjunction with results for the study reported here in which limiting iron disrupted growth and caused the death of vapA-positive and -negative strains and functional upregulation of VapA expression in vir-
ulent strains, provide strong evidence that plasmid and chromosomally encoded genes are involved in the acquisition and use of iron by \(R\) \(equi\) and that iron is important for survival and virulence of \(R\) \(equi\).

Based on the fact that adequate available iron is crucial for the survival of \(R\) \(equi\), the mechanisms by which \(R\) \(equi\) acquire iron in vivo may represent important virulence factors. Prophylactic and therapeutic strategies designed to interfere with acquisition and use of iron have proven effective in the management of other bacterial diseases.\(^4\) Individually or collectively, these potential virulence factors may provide novel targets for the development of prophylactic or therapeutic strategies to control pneumonia attributable to \(R\) \(equi\) in foals.

References


