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NOTE

Iron acquisition by *Renibacterium salmoninarum*: contribution of iron reductase

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ABSTRACT: *Renibacterium salmoninarum* is the etiologic agent of bacterial kidney disease in salmonid fish. The virulence of *R. salmoninarum* has been associated with hydrophobicity although little is known of the means by which this organism acquires iron. In this study, 7 strains of *R. salmoninarum* were grown in iron-restricted media. Siderophore production was not detected by chemical assays. The growth of all strains, but particularly non-hydrophobic strains, was considerably reduced in the presence of the high-affinity iron chelators ethylenediamine di(*o*-hydroxyphenylacetic acid) or α,α'-dipyridyl or when cultured in Chelex-treated medium. Culture supernatant from both hydrophobic and non-hydrophobic strains was found to inhibit the binding of iron by bovine transferrin and this inhibition was most pronounced in supernatants derived from iron-sufficient cultures. A strong iron reductase activity was detected in *R. salmoninarum* cells. Maximum reductase activity occurred under iron-restricted culture conditions with reduced β nicotinamide adenine dinucleotide as reductant. The results suggest that iron reductase is an important component of the iron acquisition mechanism of *R. salmoninarum*.

KEY WORDS: *Renibacterium salmoninarum* · Iron acquisition · Iron reductase

Renibacterium salmoninarum is a Gram-positive bacterium which causes a chronic granulomatous infection, bacterial kidney disease (BKD), of salmonid fish (Evelyn 1993). There is considerable evidence to suggest that the organism is an obligate, intracellular pathogen and the pathogenesis of BKD is closely associated with intracellular survival and multiplication within salmonid phagocytes (Evenden et al. 1993). *R.salmoninarum* is fastidious and slow growing and there is a paucity of knowledge of the factors which assist and promote BKD infection. The virulence of strains has been associated with autoagglutination and hydrophobicity (Bruno 1988).

The ability of an invading pathogen to multiply successfully within the host environment is a commonly shared and essential factor in all infections. Here, the complex interactions with the host may elicit physiological changes within the pathogen growing *in vivo* which may not be reflected during *in vitro* growth. In this respect the availability of iron may be vital because it is essential for bacterial growth and replication. The ability to obtain iron *in vivo* is an important virulence determinant for many microbes including intracellular bacterial pathogens such as *Listeria monocytogenes* (Sword 1966, Alford et al. 1991) and *Legionella pneumophila* (Byrd & Horwitz 1989). Since iron is not normally freely available in body tissues and fluids, microorganisms have evolved diverse mechanisms for acquiring iron *in vivo* including the production of siderophores that can remove iron from transferrin or cell-derived sources (Brock et al. 1991), the direct binding of transferrin, lactoferrin or haem on the bacterial cell surface (Blanton et al. 1990, Otto et al. 1990) and the reduction of ferric iron to ferrous complex which may be readily bound and transported by the bacteria (Cowart & Foster 1985, Adams et al. 1990, Johnson et al. 1991).

Studies of the nutritional requirements of *Renibacterium salmoninarum* have led to the development of complex and semi-defined media (Evelyn 1977, Embley et al. 1982). However, to date no information exists regarding the means by which *R. salmoninarum* acquires iron. The purpose of this study was to investigate the mechanism of iron acquisition *in vitro* by *R. salmoninarum*.

Materials and methods. Six isolates of *Renibacterium salmoninarum* including the type strain

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Table 1. Source and origin of *Renibacterium salmoninarum* strains used in this study

Strain	Other strain numbers	Hydrophobicity/ autoagglutination	Fish source	Origin
ATCC33209	MT 251	+	*Oncorhynchus tshawytscha*	USA
MT 414	MT 239	-	*Salmo salar*	Scotland
MT 417	MT 241	-	*Oncorhynchus kisutch*	Scotland
MT 420		+	*Salmo salar*	Scotland
MT 425		+	*Oncorhynchus mykiss*	Scotland
MT 452	222/88	-	*Oncorhynchus mykiss*	Scotland
910019		+	*Oncorhynchus mykiss*	England

(ATCC33209, Table 1) have been previously described (Bruno & Munro 1986). Isolate 910019 from an outbreak of BKD in 1991, and *Aeromonas salmonicida* CM30 were provided by Dr C. J. Rodgers, Ministry of Agriculture, Food and Fisheries, Fish Disease Laboratory, Weymouth, Dorset, England. Three isolates were non-autoagglutinating and non-hydrophobic and have been shown to be of low virulence and to lack a 57K saline extractable cell-associated protein which has been associated with virulence (Bruno 1988, 1990). The remaining 4 isolates were autoagglutinating and hydrophobic. Tests for autoagglutination, hydrophobicity and biochemical properties were carried out as described by Bruno (1988) and Bruno & Munro (1986).

Renibacterium salmoninarum was maintained on Mueller-Hinton agar (MHCA) or broth (MHCB) supplemented with 0.1% (w/v) L-cysteine hydrochloride at 15°C. Growth of bacterial cells was assessed using the methods described by Embley et al. (1982). Every manipulation involved 2 checks for contaminating organisms: (1) Gram's stain, and (2) spread plating on MHCA and incubation at 15°C followed by Gram's stain of the subsequent growth. In addition, at the end of experimental work, Western blots of cell lysates of all cultures were probed with rabbit anti-*R. salmoninarum* strain ATCC33209 antiserum and showed good immunological recognition of all *R. salmoninarum* strains used. The availability of iron in MHCA and MHCB was restricted by the addition of either ethylene diamine di(*o*-hydroxyphenylacetic acid) (EDDHA), α,α'-dipyridyl (DPD) or by the pretreatment of MHCB with Chelex 100 (BioRad). Chelex 100 resin is a chelating ion exchange resin which possesses a very high preference for metal cations, particularly divalent species such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+} and also Fe^{3+}. Culture media which had been treated with Chelex 100 resin were therefore depleted of various metals including iron, although only the iron content of the media was assayed. For treatment with Chelex 100, 5 g of resin was added for each 100 ml of MHCB and stirred gently for 1 h. The broth was filtered to remove the resin and then autoclaved. EDDHA and DPD were prepared as 10 mM solutions and filter-sterilised prior to addition to autoclaved media. Preliminary experiments were carried out on MHCA using concentrations of 10 to 250 µM EDDHA and 10 to 125 µM DPD in order to define minimum inhibitory concentrations of each chelator. On the basis of this work 200 µM EDDHA and 100 µM DPD were chosen as suitable working concentrations. The iron content of the various media was determined by the ferrozine assay described by Stookey (1970). Concentrations of 200 µM EDDHA, 100 µM DPD or Chelex treatment reduced the free iron content of the media from in excess of 200 µg l^{-1} to less than 40 µg l^{-1}. To minimise the storage of intracellular ferric iron, bacteria were passaged twice in iron-restricted MHCB (10 µM EDDHA or DPD) for 10 d before use. It was observed that continuous culture of *R. salmoninarum* on iron-restricted media with higher chelator concentrations led to non-viability. All glassware was washed in 6 M HCl and rinsed 10 times in deionised-distilled water from a Milli Q purification system (Millipore) to remove contaminating iron. Contaminating iron was removed from EDDHA by the method of Rogers (1973).

The following compounds were tested for the ability to restore growth of *Renibacterium salmoninarum* cultured under iron-restricted conditions: bovine haemin, bovine haemoglobin, bovine apotransferrin, bovine holotransferrin, ferric citrate, ferric chloride and ferrous sulphate. All solutions were prepared fresh and used at final concentrations of 100 and 200 µM. The bacteria were washed in buffered saline and 10^5 cells were inoculated on MHCA plates containing either 250 µM EDDHA or 125 µM DPD. Paper discs containing 50 µl of the iron compounds were placed on the agar plates.

Assays for catecholate and hydroxymate siderophore activity were carried out every 2 d up to 3 wk post-inoculation and then weekly up to 13 wk post-inoculation. Milli Q water was used throughout. Colorimetric assays were used to assess the possible production of phenolate-catechol siderophores using 2,3-dihydroxybenzoic acid (2,3-DHBA) as standard (Arnow 1937,

Rioux et al. 1983) and hydroxamate siderophores using hydroxylamine monohydrochloride as standard (Atkin et al. 1970, Arnold & Viswanatha 1983). The chrome azurol S (CAS) universal chemical assay for siderophore detection was also used (Schwyn & Neilands 1987). Iron reductase activity was measured by using the ferrous iron chelator 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine, usually called ferrozine (Dailey & Lascelles 1977). The reaction mixture contained, in a final volume of 2 ml, either 50 µg of sonicated cell extract protein or 10 µg of culture supernatant protein, 5 µM of ferrozine in 0.01 M Tris-HCl, pH 7.6, 100 µg of ferric citrate (pH 7.0), 2 µM of either reduced β nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 0.01 M Tris-HCl, pH 7.6. Cuvettes were filled with nitrogen gas and sealed. The reactions were carried out in polystyrene cuvettes at room temperature and the change in optical density was monitored at 562 nm. The reference cuvettes contained all of the reaction components except reductant. The reductase activity was recorded as the change in optical density over time. Specific activity of the cell sonicates was calculated as the amount of product formed mg protein^{-1} h^{-1}.

The iron binding activity of cell-free culture supernatant of *Renibacterium salmoninarum* cultured for 3 wk was determined by the method of Norrod & Williams (1978). Either 0.2 ml of water, uninoculated culture medium (with or without chelator) or *R. salmoninarum* culture supernatant was added to 0.8 ml of iron-free bovine transferrin solution (2.5 mg ml^{-1} in 0.1 M Tris-HCl, pH 7.4) and incubated at room temperature (22°C) for 1 h. Absorbance at 470 nm was recorded as an indication of the binding of iron by transferrin after each addition of 5 µl of 1 mM ferric nitrilotriacetate, an iron compound that reacts stoichiometrically with transferrin. Bovine apotransferrin contained less than 0.002% iron as determined by the manufacturer (Sigma).

Bacterial cultures grown for 3 wk were centrifuged (4000 × *g*, 4°C, 20 min), the supernatant retained and the cells resuspended in 5% of the original volume in 10 mM Tris-HCl, pH 7.4. Bacterial suspensions were disrupted by sonication (W-385 Ultrasonic Processor, Heat Systems Ultrasonics) on ice (5 s cycle, 50% duty cycle, 20 min). Unbroken cells and debris were removed by centrifugation (9000 × *g*, 4°C, 15 min) and the protein concentration of the resultant cell lysates and the culture supernatants was estimated by the method of Bradford (1976) using a commercial assay kit following the manufacturer's instructions (BioRad).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (LKB 2000) using a 4% acrylamide stacking gel and 10% separating gel (Laemmli 1970). For the detection of free aldehydes in SDS-PAGE gels of *Renibacterium salmoninarum* cell sonicates, gels were fixed in 7% (vol/vol) acetic acid (24 h, room temperature, constant shaking), oxidized in 0.2% (wt/vol) periodic acid (45 min, 4°C) and then immersed in Schiff's reagent (45 min, 4°C). Gels were destained in 10% (vol/vol) acetic acid until the red bands indicative of reducing sugar moieties were visible.

Comparison of paired groups or data sets was carried out using Student's *t*-test for the difference of means (paired samples) as described by Wardlaw (1987). All experiments were carried out 3 times except the addition of iron-containing compounds which was carried out twice.

Results and discussion. Restricting the availability of iron reduced the growth of *Renibacterium salmoninarum*, although non-autoagglutinating strains were much more sensitive to iron-restricted conditions than autoagglutinating strains (Fig. 1). The ferrous iron chelator DPD was more effective in restricting the growth of autoagglutinating strains in MHCB than either Chelex treatment or EDDHA. This may be a

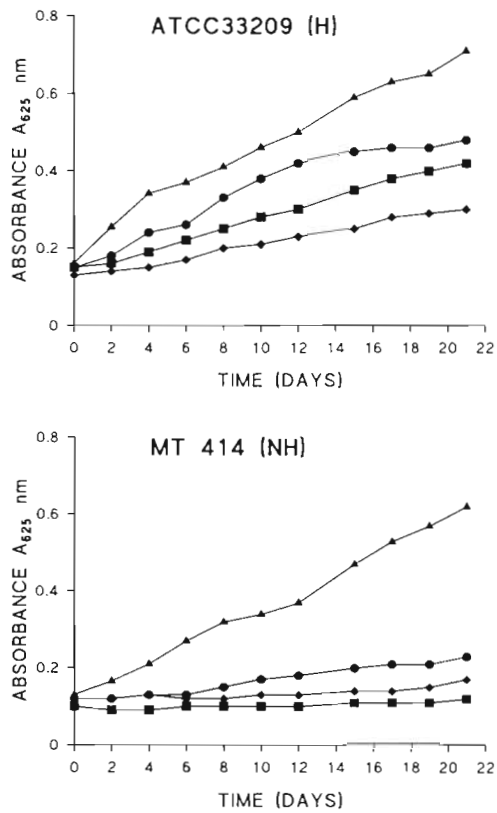

Fig. 1 Growth characteristics of *Renibacterium salmoninarum* in (▲) MHCB; (◆) MHCB + 100 µM DPD; (●) MHCB + 200 µM EDDHA or (■) Chelex 100 treated MHCB. H: hydrophobic, autoagglutinating; NH: non-hydrophobic, non-autoagglutinating

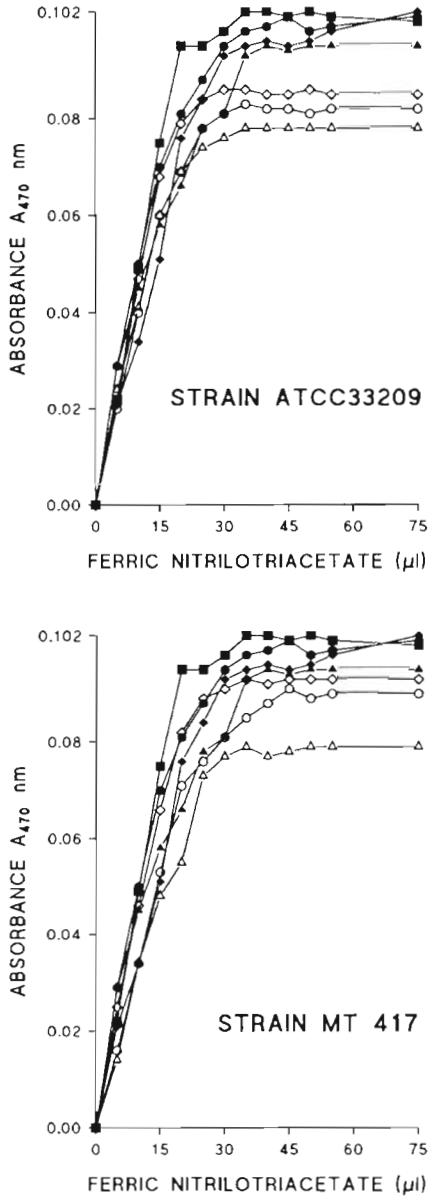

Fig. 2. Effect of culture supernatant from *Renibacterium salmoninarum* strains ATCC33209 (hydrophobic) and MT 417 (non-hydrophobic) on the binding of iron by iron-free transferrin. (■) Milli Q water; (▲) MHCB; (◆) MHCB + 100 µM DPD; (●) MHCB + 200 µM EDDHA; (△) iron-sufficient culture supernatant; (◇) 100 µM DPD iron-restricted culture supernatant; (○) 200 µM EDDHA iron-restricted culture supernatant. Graphs representative of results obtained for all 6 strains of *R. salmoninarum* are presented

consequence of the accumulation of DPD within the bacterial cells which were observed to turn bright pink as the cells multiplied. It was also observed that continued culture of *R. salmoninarum* on iron restricted media led to non-viability. Only ferrous sulphate and ferric citrate were found to be able to stimulate the growth of *R. salmoninarum* in the presence of inhibitory concentrations of EDDHA and DPD as shown by zones of growth around the iron-containing discs.

There was no evidence for the production of phenolate-catechol and hydroxymate siderophores in the culture supernatants of strains of *Renibacterium salmoninarum* grown under conditions of iron-restriction. Supernatants from 13 wk cultures were also assayed after 20-fold concentration by freeze-drying, again with no success. Strain 910019 was not included in these assays. In a control group, the culture supernatant of *Aeromonas salmonicida* CM30 grown in tryptone soy broth with 277 µM EDDHA was positive for phenolate and produced strong orange zones surrounding colonies on CAS agar. No evidence for siderophore production by *R. salmoninarum* could be found using the CAS test and it was necessary to use a heavy inoculum of culture in order to obtain growth.

The ability of culture supernatant to inhibit the binding of ferric nitrilotriacetate to iron-free bovine transferrin was examined using spectrophotometry. When uninoculated culture medium, either with or without chelator, was present in the assay some inhibition of the binding of iron compared with samples containing only Milli Q water was observed. However, when compared with their respective controls, the culture supernatants of all strains did have some ability to prevent the binding of iron to iron-free transferrin (Fig. 2). This inhibition, although present in iron-restricted culture supernatants ($p < 0.05$), was most pronounced in supernatants from iron-sufficient cultures ($p < 0.0001$) and cannot therefore be attributed to a siderophore. The culture supernatants may have contained a protease capable of cleaving transferrin or possessed some other product that binds non-specifically to the iron binding site on the transferrin molecule.

Autoagglutinating and non-autoagglutinating strains of *Renibacterium salmoninarum* produce an iron reductase (Table 2). Generally, regardless of the availability of iron, non-autoagglutinating strains possessed a higher level of enzyme activity than autoagglutinating strains, being an average of 20 to 64% greater ($p < 0.05$) using either NADH or NADPH as the reductant. For all strains, when NADH was used as the reductant the specific activities of sonicated cell extracts were from 12 to 174% higher than those recorded for NADPH ($p < 0.01$). Under iron-restricted culture conditions, all strains showed increased reductase activity which varied from 30 to 80% for NADH and 3.5 to 61% for NADPH ($p < 0.05$). Only low levels (specific activity <5) of iron reductase activity were detected in *R. salmoninarum* culture supernatants.

In an attempt to identify cell-associated components from *Renibacterium salmoninarum* which were regulated by the availability of iron cell extracts were pre-

pared and separated by SDS-PAGE. Gels stained with Coomassie Blue did not reveal the presence of any obvious additional proteins in extracts derived from iron-restricted cultures. However, SDS-PAGE gels stained with periodic acid-Schiff's reagent (PAS) consistently revealed the presence of material at the top of the gel as well as several components ranging in molecular mass from 140 to 200 kDa which were present only in cell extracts from iron-restricted cultures (Fig. 3). The nature of the iron regulated components identified in *R. salmoninarum* has yet to be elucidated. Nevertheless, their exclusive production under iron-restricted conditions by all strains of *R. salmoninarum* examined in this work suggests they may perform a basic and essential function related to iron acquisition (Brown & Williams 1985).

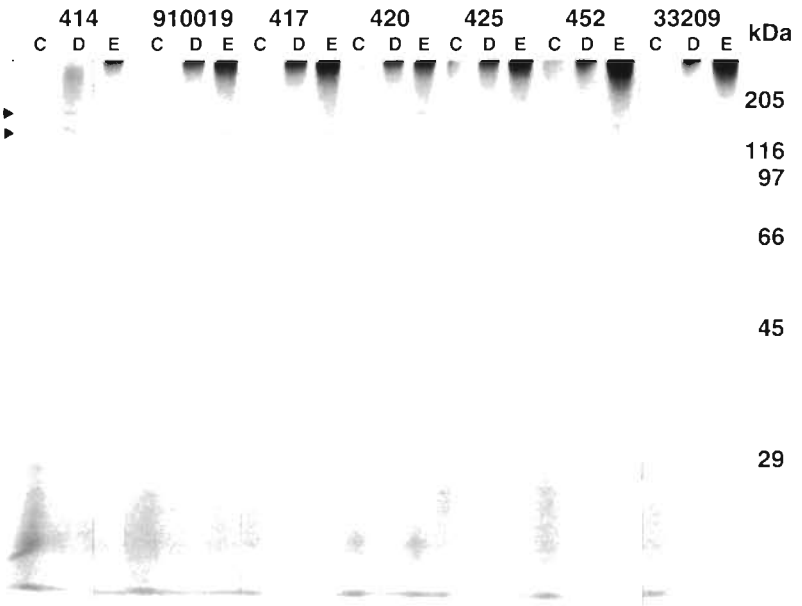

Fig. 3. Detection of iron-regulated carbohydrates in cell extracts of 7 *Renibacterium salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency. Track C: cultures grown in MHCB. D: cultures grown in MHCB with 100 µM DPD. E: cultures grown in MHCB with 200 µM EDDHA. Positions of protein molecular weight standards are indicated. Relevant strain numbers appear above each set of samples (see Table 1). About 25 µg protein was separated in each lane. Arrows mark the positions of the carbohydrates which were unique to strains grown under iron-restricted conditions

An absence of siderophores has been noted in a variety of bacteria including the intracellular pathogens *Legionella pneumophila* (Reeves et al. 1983) and *L. monocytogenes* (Cowart & Foster 1985). Some pathogens possess specific cell surface receptors for transferrin and lactoferrin (Blanton et al. 1990). Whether *Renibacterium salmoninarum* is able to bind and utilise fish transferrin, lactoferrin or heme has yet to be investigated, although bovine transferrin, haemin and haemoglobin were unable to reverse the growth inhibition induced by EDDHA, DPD and Chelex in this study.

Iron reductase enzymes have been shown to be important in the ability of many bacteria to acquire iron from siderophores. The reductase acts to reduce the iron in the ferrisiderophore chelate from Fe^{3+} to Fe^{2+}. Since Fe^{2+} has a lower affinity for the siderophore, the Fe^{2+} is released and can be utilised by the bacterium. In addition, there is evidence that some iron reductase enzymes may be involved in the acquisition of iron from ferric complexes other than ferrisiderophore chelates (Cox 1980, Huyer & Page 1989, Johnson et al. 1991). Recent studies of *Listeria monocytogenes* have identified a soluble iron reductase protein of 8 to 10 kDa molecular mass which is present in culture supernatants and acts to rapidly reduce iron from transferrin (Cowart & Foster 1985, Adams et al. 1990). Johnson et al. (1991) have recently identified a cell-

Table 2. Specific activity of iron reductase in cell lysates of *Renibacterium salmoninarum* strains with NADH or NADPH as reductant. Specific activity is expressed as nM Fe^{2+} formed mg protein^{-1} h^{-1} calculated as $\Delta A\ h^{-1} \times \varepsilon^{-1} \times$ vol (l) $\times 10^9 \times$ protein concentration (mg), where A is absorbance and ε is the extinction coefficient. Figures represent the mean of 3 readings ± standard error. *Hydrophobic, autoagglutinating strains; non-hydrophobic, non-autoagglutinating strains are unmarked

Strains	NADH		NADPH	
	Iron-restricted	Iron-sufficient	Iron-restricted	Iron-sufficient
ATCC33209*	156.99 ± 3.42	92.34 ± 3.82	88.89 ±3.00	82.29 ±5.38
MT 414	208.36 ± 8.97	115.41 ± 6.53	106.09 ±5.82	99.64 ±2.49
MT 417	245.16 ±10.98	164.87 ± 8.14	104.66 ±4.80	69.54 ±3.61
MT 420*	140.86 ± 7.87	90.86 ± 6.33	80.29 ±3.46	77.60 ±4.97
MT 425*	137.63 ± 7.76	105.77 ± 4.21	74.91 ±4.27	57.17 ±1.57
MT 452	258.78 ± 8.69	162.01 ±10.31	122.43 ±4.75	75.97 ±3.57
910019*	169.17 ± 4.98	98.62 ± 7.05	61.65 ±2.77	56.38 ±3.25

associated ferric citrate reductase which is important in the acquisition of iron by *Legionella pneumophila*. They found that compared to virulent *L. pneumophila* cells, avirulent cells showed a higher reductase activity which was maximal with NADPH as reductant. Both Cowart & Foster (1985) and Johnson et al. (1991) reported that avirulent strains of *L. monocytogenes* and *L. pneumophila* respectively have a higher iron requirement than virulent strains. They suggest that, given the limitations of iron availability in the intracellular environment and the documented effects of iron deprivation on the intracellular growth of both these pathogens (Byrd & Horwitz 1989, Alford et al. 1991), the inability of avirulent cells to survive within the phagocytic cell may be in part a result of the higher iron requirement of avirulent cells.

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