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Porphyromonas gingivalis Ferrous Iron Transporter FeoB1 Influences Sensitivity to Oxidative Stress▽

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Porphyromonas gingivalis **FeoB1 is a ferrous iron transporter. Analysis of parental and *feoB1*-deficient strains of the periodontal pathogen revealed that the *feoB1*-deficient mutant strain had an increased ability to survive oxidative stress. Specifically, survival of the mutant strain was increased 33% with exposure to peroxide and 5% with exposure to atmospheric oxygen compared to the parental strain. Interestingly, the ability to survive intracellularly also increased fivefold in the case of the *feoB1*-deficient mutant. Our data suggest that although the FeoB1 protein is required for ferrous iron acquisition in *P. gingivalis*, it also has an adverse effect on survival of the bacterium under oxidative stress conditions. Finally, we show that *feoB1* expression is not iron dependent and is dramatically reduced in the presence of host cells, consistent with the observed deleterious role it plays in bacterial survival.**

Iron is an indispensable nutrient for most organisms. As a cofactor for multiple enzymes, such as superoxide dismutase, catalase, peroxidase, Dps, and rubrerythrin (13, 17, 19), iron plays a significant role in a variety of processes in the bacterial cell, including oxidative stress. On the other hand, iron is also an indispensable cofactor for generation of oxygen radicals from peroxide and, as such, contributes significantly to oxidative stress encountered by bacteria. Given both the positive and negative effects of iron, levels within the cell must be regulated tightly to ensure adequate iron for bacterial growth while preventing accumulation of free iron, which would then be available for interaction with peroxide and generation of hydroxyl radicals.

Iron is not usually encountered in its free form in the human host due to its toxic nature; most iron is bound in hemoglobin. Other iron sources include transferrin and lactoferrin. Since iron is not freely available for bacterial uptake, specialized mechanisms capable of extracting the iron from host proteins and then transporting the iron into the microbial cell are necessary. The typical transporters in Gram-negative bacteria include high-affinity outer membrane receptors and inner membrane transporters (14).

Porphyromonas gingivalis, an established periodontopathogen, requires iron for growth (3). The bacterium lacks members of the protoporphyrin IX synthetic pathway but requires hemin as a cofactor for fumarate reductase and cytochromes, so it must acquire this nutrient from the environment. The strict requirement for iron and hemin may explain the multiple hemin/iron uptake mechanisms encoded in the genome of *P. gingivalis* W83. *P. gingivalis* cells cultured on blood agar plates show black pigmentation, highlighting the importance and accumulation of hemin. Hemin uptake mechanisms include the *hmu*, *iht*, and *tlr* loci (1, 5, 12, 14, 16). Although the *hmu* locus has been demonstrated to be the major hemin uptake locus in *P. gingivalis* (12), mutants deficient in this locus can still acquire hemin, indicating that the other hemin uptake loci are functional in this bacterium. In addition to the hemin uptake loci, a ferrous uptake transporter was recently demonstrated to be both present and indispensable for iron acquisition in *P. gingivalis* (4). Mutants deficient in the transporter had reduced iron levels compared to those in the parental strain, showing that iron transport was abolished in the mutant strain (4). However, despite demonstrating its role in iron uptake, only limited investigation of the role of FeoB1 in oxidative stress sensitivity was reported. Given the harmful nature of iron, we hypothesized that the presence of the transporter would increase the sensitivity of bacteria to oxidative stress and ultimately affect microbial survival in the presence of host cells. To address these issues, we examined the role of the FeoB1 protein in oxidative stress protection and also investigated the ability of *P. gingivalis* to survive within epithelial cells. We also examined the roles of metals, oxygen, and exposure to host cells in expression of the FeoB1 transporter to gain insight into the molecular basis of the mechanisms of iron homeostasis regulation in *P. gingivalis*. The results of our studies contribute to a more comprehensive understanding of the role of iron in oxidative stress in *P. gingivalis* and also shed light on the role of microbial iron transporters in host-pathogen interactions.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Porphyromonas gingivalis* strain W83 was used in our study. The strain was cultured in an anaerobic atmosphere composed of 10% H_2, 10% CO_2, and 80% N_2 at 37°C. For maintenance, blood agar plates (TSA II, 5%

TABLE 1. Strains and plasmids used in this study

Strain	Plasmid	Description	Source or reference
P. gingivalis strains			
W83		Parent strain	11
V2897		Em^r; FeoB1-deficient mutant	This study
E. coli strains			
One Shot		Chemically competent cells	Invitrogen
Top 10	pCR 2.1	Cloning vector	Invitrogen
V2198	pVA2198	Carries 2-kb *ermF-ermB* cassette	6
V2762	pVA2762	Ap^r; pCR 2.1 containing the 2.2-kb *feoB* fragment PCR amplified from *P. gingivalis* W83 genomic DNA	This study
V2760	pVA2760	Ap^r Em^r; pVA2762 with the *ermF-ermB* cassette inserted at the SmaI site	This study

sheep blood) (BBL, Cockeysville, MD) were used, and liquid cultures were prepared in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) supplemented with hemin (5 μg/ml) (Sigma, St. Louis, MO), yeast extract (5 mg/ml), cysteine (1 mg/ml) (Sigma, St. Louis, MO), and vitamin K_3 (1 μg/ml) (Sigma, St. Louis, MO). Growth studies were done by serial passage (daily subculture using a 1:10 dilution) in BHI medium depleted of divalent and transition metals by use of Chelex 100 resin (chelating ion-exchange resin; Bio-Rad, Hercules, CA) and supplemented with $MgCl_2$, $CaCl_2$, $ZnCl_2$ (100 μM for each metal), and protoporphyrin IX (PPIX) (5 μg/ml).

Clindamycin (0.5 μg/ml) was used for selection and maintenance of a *P. gingivalis feoB1* mutant containing the *ermF-ermB* cassette (6).

Escherichia coli was grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar. Carbenicillin (50 μg/ml) and erythromycin (300 μg/ml) were used for selection of strains.

Construction of *P. gingivalis feoB1* mutant strain. The primers used in this study are listed in Table 2. A fragment of *P. gingivalis* W83 genomic DNA encoding the entire FeoB1 protein was PCR amplified and cloned into the pCR2.1 vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). An *ermF-ermB* cassette was inserted into the SmaI site, located at bp 190 of the cloned *feoB1* gene. The mutagenized *feoB1* linear fragment was released by digestion with EcoRI and used to electroporate *P. gingivalis* W83 according to a previously published protocol (6). Transformants were selected on BHI agar plates supplemented with clindamycin (0.5 μg/ml). The expected insertion of the *ermF-ermB* cassette into the genome of the mutant strain was confirmed by PCR. The mutant strain was designated V2897.

TABLE 2. Primers used in this study

Primer	Position in *feoB1* (bp)	Sequence (5′–3′)
Primers for *feoB* amplification		
FeoF		GATATCGGATCCATGATGAAAAAGAGTGCTAC[a]
FeoR		GCAAGCGAGCTCTCCTCCGACCAACCGCCATAC[b]
Primers for mutant verification		
FeoBF	215–235	CCGAAGGTGCATTCTCGTATG
FeoBR	632–612	CAGTACCGATACTTGTCACTC
Primers for qRT-PCR		
FeoB1-F		GAAAGCATTATCACGGATCAG
FeoB1-R		TGTAGCTGCTCCGGTATGTTTCT

[a] The BamHI site is underlined.
[b] The XhoI site is underlined.

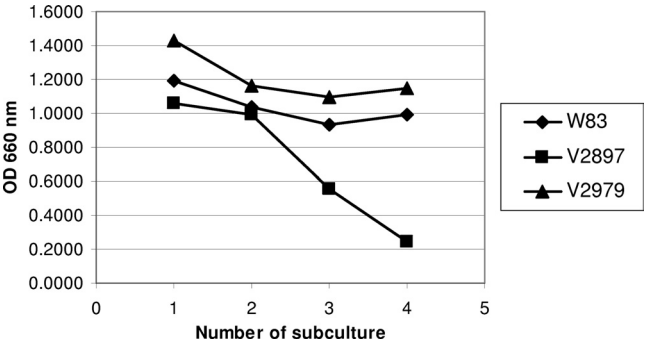

FIG. 1. Growth of *P. gingivalis* strains. *P. gingivalis* strains were subcultured daily in iron-depleted medium. Growth was monitored by measuring the OD_{660} of the culture.

Complementation of the *feoB1* mutation. The disrupted *feoB1* gene in V2897 was restored by conjugation. Briefly, 100 μl of heat-killed *P. gingivalis* W83 cells (prepared by incubation at 65°C for 30 min) was mixed with 100 μl of *P. gingivalis* V2897 (*feoB1* mutant). Following mixing of the two cell types, 1 ml of prereduced BHI medium was added, and the mixture was incubated anaerobically at 37°C for 18 h. Following 1:10,000 dilution with BHI, 200 μl of the coculture was spread on blood agar plates and incubated anaerobically at 37°C for 7 days. As a control for viability loss, the heat-killed bacteria were plated in parallel to ensure that no bacterial growth was observed. The genetic organization of the *feoB1* locus in resulting colonies was examined by PCR with primers designed for both ends of the *feoB1* gene. Colonies containing the parental version of the *feoB1* gene were further examined for the ability to grow on blood agar plates supplemented with clindamycin (0.5 μg/ml). A clindamycin-sensitive colony containing the intact version of the *feoB1* gene was designated the complemented strain and was called strain V2979.

Metal uptake studies. Metal uptake studies were done as described previously (7). Briefly, *P. gingivalis* cells were depleted of divalent and transition metals by serial passage in BHI medium treated with Chelex 100 chelating ion-exchange resin (Bio-Rad, Hercules, CA) and supplemented with $MgSO_4$, $CaCl_2$, $ZnCl_2$ (100 μM for each metal), and PPIX (5 μg/ml). The cells were harvested, and *Mycoplasma* broth depleted of metal ions by use of Chelex resin and adjusted to pH 6.5 was used in transport studies. Metal uptake was done using $^{55}Fe^{2+}$ and $^{54}Mn^{2+}$ (Perkin-Elmer, Boston, MA). The studies were performed using a MultiScreen assay system (Millipore, Bedford, MA). *P. gingivalis* cells (50 μl; 2×10^7 cells) were added to wells of 96-well clear plates with a Hydrophil polyvinylidene difluoride (PVDF) Durapore membrane (Millipore, Bedford, MA) prewetted with 150 μl of Chelex-treated mycoplasma. $^{55}FeCl_2$ was then added to the wells to a final concentration of 0.46 μM. The plate was incubated anaerobically at 37°C for various times ranging from 1 min to 20 min. Energy-independent binding and uptake were assessed by performing the assay with prechilled cells and materials. Following incubation, liquid was removed by vacuum aspiration and the plates were washed two times with TBS (125 mM NaCl, 25 mM Tris, pH 8.0) supplemented with Tween 20 (0.1%), followed by one wash in TBS. Air-dried filters were isolated with a MultiScreen punch (Millipore, Bedford, MA). Beta radiation was detected with channel 350, using a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Active uptake was determined by subtracting count-per-minute values from experiments done on ice from those for experiments performed at 37°C.

Manganese uptake was determined as described above, except that the cells were incubated aerobically at ambient temperature in the presence of 0.05 μM $^{54}Mn^{2+}$. Experiments performed on ice served as controls for energy-independent binding and uptake. Gamma radiation was detected using channel 850 (10).

Sensitivity of *P. gingivalis* to air with and without hydrogen peroxide. Mid-logarithmic-phase cultures of *P. gingivalis* were divided into three aliquots. The first aliquot served as a control and was incubated anaerobically at 37°C. The second was cultured with agitation (200 × *g*) aerobically at 37°C. The final was grown with agitation (200 × *g*) aerobically at 37°C in the presence of 0.5 mM hydrogen peroxide. Culture aliquots were collected at various time intervals, transferred to an anaerobic chamber, serially diluted, and then plated on blood agar plates. Following anaerobic incubation at 37°C for 7 days, the number of colonies was determined.

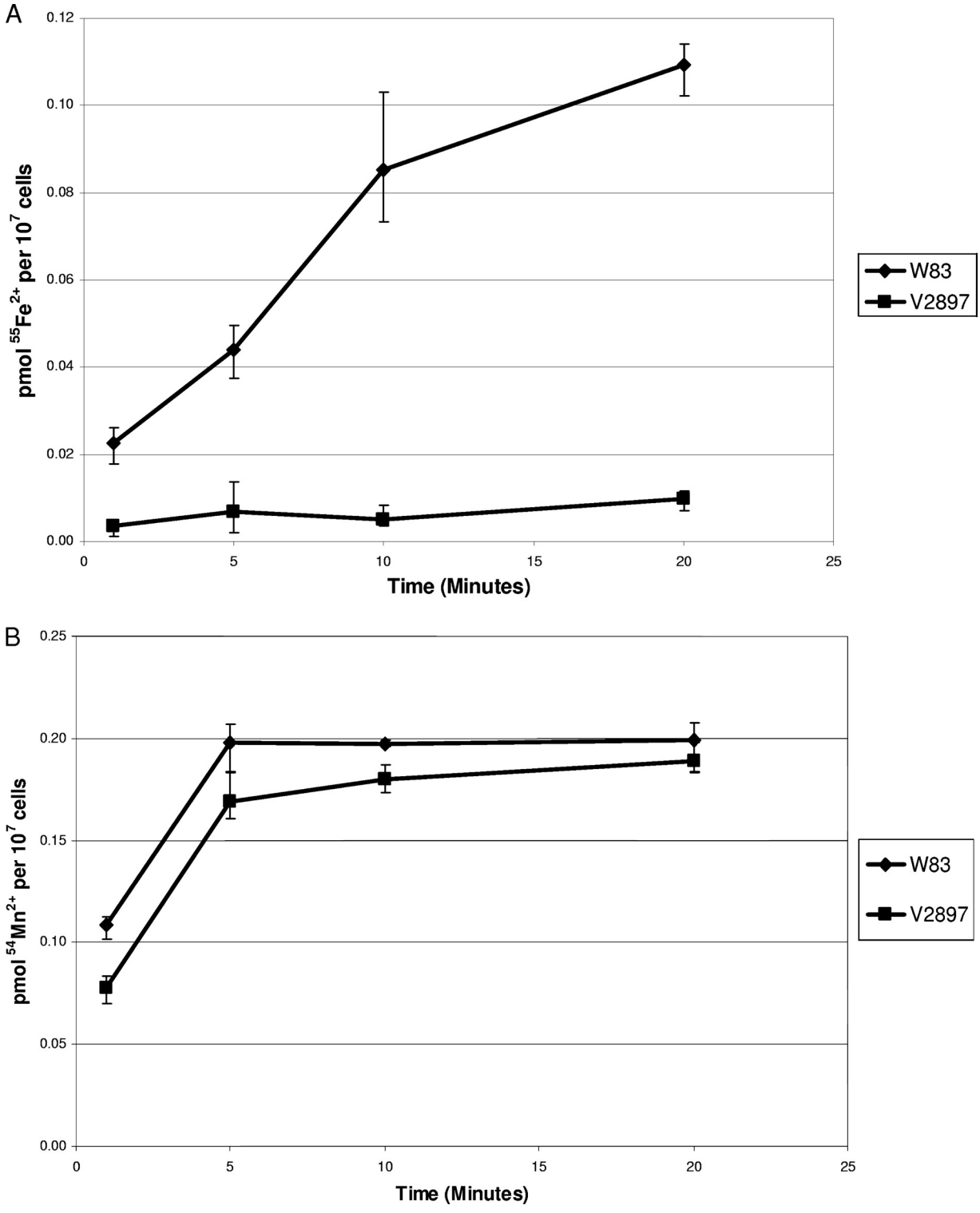

FIG. 2. Metal uptake in *P. gingivalis* strains. (A) Active uptake of $^{55}Fe^{2+}$ in strains W83 (parental strain) and V2897 (*feoB1* mutant) was determined under anaerobic conditions, using 0.46 μM radiolabeled iron. (B) Active uptake of $^{54}Mn^{2+}$ was examined using 0.05 μM radiolabeled manganese. Experiments were performed in triplicate.

Sensitivity of *P. gingivalis* to hydrogen peroxide was also examined using disc diffusion assays done on blood agar plates. Sterile discs saturated with 5% or 15% hydrogen peroxide were then placed on the surfaces of the bacterium-containing blood agar plates. Following incubation under anaerobic conditions at 37°C for 7 days, the microbial growth inhibition zones around the discs were measured.

Survival with host cells. Survival studies were done as described previously (7, 19). Briefly, HeLa cells were infected with *P. gingivalis* strains at a multiplicity of

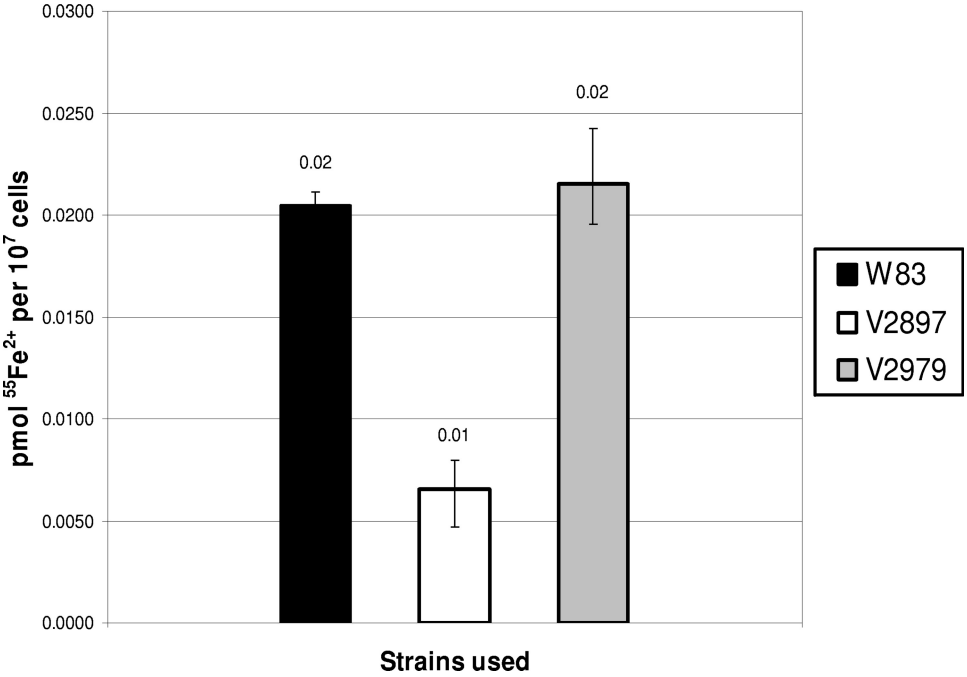

FIG. 3. Iron uptake is restored in FeoB1-complemented strain. Active uptake of $^{55}Fe^{2+}$ in strains W83 (parental strain), V2897 (*feoB1* mutant), and V2979 (complemented strain) was examined for 20 min under anaerobic conditions. The experiment was performed in triplicate.

infection (MOI) of 100. Plates were incubated anaerobically at 37°C for 30 min. After being washed, cells were disrupted by three cycles of freeze-thawing and then diluted 4:1 with BHI medium. Finally, the mixture was plated on blood agar plates, and viable bacteria were estimated as the number of colonies formed after 11 days of incubation under anaerobic conditions. To determine the number of bacteria surviving intracellularly following washing in cell medium, extracellular bacteria were killed by incubation of the infected cells with medium supplemented with gentamicin (300 μg/ml) and metronidazole (400 μg/ml) for 1 h. Washed cells were then disrupted as described above, and the number of colonies was determined by culturing of the diluted mixture on blood agar plates, anaerobically, at 37°C.

Confocal microscopy. Cells were prepared as described previously (7, 19) and analyzed under a confocal laser scanning microscope (LSM510; Carl Zeiss, Bannockburn, IL).

Exposure of *P. gingivalis* to host cell-conditioned medium. To prepare conditioned medium, HeLa cells were cultured overnight in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine without antibiotics. The medium was harvested and used to culture *P. gingivalis* W83. For the control, DMEM–10% FBS medium that was not exposed to host cells was used to grow bacteria. Actively growing cells from an overnight culture of *P. gingivalis* W83 in BHI were diluted 10-fold and then cultured and grown to early logarithmic phase (optical density at 660 nm [OD_{660}] = 0.3). The cells were pelleted, washed in phosphate-buffered saline (PBS), and divided into two aliquots. One aliquot was suspended in host cell-conditioned medium, and the other, serving as a control, was added to unconditioned medium. The *P. gingivalis* suspensions were then grown anaerobically at 37°C. Culture aliquots were removed at various times postincubation, and the cells were harvested and then stored at −70°C prior to RNA isolation.

Exposure of *P. gingivalis* to oxidative stress and a metal chelator. An overnight culture of *P. gingivalis* W83 was diluted to an OD_{660} of 0.2 with anaerobic BHI and incubated anaerobically until the culture reached mid-logarithmic phase (OD_{660} = 0.5). The culture was divided into several aliquots; one aliquot was incubated anaerobically and served as a control, the second was supplemented with 5 mM EDTA, and the third was grown in the presence of 200 μM hydrogen peroxide. As described above, culture aliquots were removed at various time points postincubation, and bacterial cells were harvested and stored at −70°C prior to RNA isolation.

RNA isolation and quantitative reverse transcription-PCR (RT-PCR) analysis. Total cellular RNA was isolated from *P. gingivalis* cultures grown in BHI under various conditions (with and without iron, aerobically [in the presence of 6% oxygen] and anaerobically, in conditioned and unconditioned medium), using an RNeasy Mini kit according to the manufacturer's (Qiagen, Valencia, CA) instructions. Contaminating DNA was removed by treatment of the preparations with RNase-free DNase (Qiagen). Any remaining DNA was removed by additional treatment with RNase-free DNase (Ambion, Austin, TX) at 37°C for 30 min. Total RNA preparations were stored at −80°C.

Real-time quantitative RT-PCR was done using a SYBR green-based detection system on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA). Primers (Table 2) were designed using Primer 3 software (Source Forge [http://frodo.wi.mit.edu]). The quality and specificity of amplified products were confirmed by visualization on a 2% agarose gel. A calibration curve was constructed using serial fivefold dilutions of 100 ng of total RNA. Experimental samples were tested in triplicate under the conditions recommended by the manufacturer, using 20 ng of total RNA (except for 16S rRNA, in which case we used 0.2 ng of total RNA). Reverse transcriptase was omitted for samples serving as negative controls. cDNA was synthesized with BioScript reverse transcriptase (Bioline, Taunton, MA) at 42°C for 90 min. The quantitative PCR was done using a thermal profile consisting of 1 cycle of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. To control for RNA quantity, a probe specific for 16S rRNA was used.

RESULTS

Growth of *feoB1* mutant strain. The FeoB1-deficient mutant grew to levels comparable to those of the parental strain under iron-replete conditions (results not shown). However, drastically reduced growth potential was observed under iron-depleted conditions compared to the W83 strain and a strain in which the gene was restored, indicating a major role of the protein in iron uptake (Fig. 1).

FeoB1 is required for ferrous iron transport but has no role in manganese acquisition. Previous studies showed that FeoB1 plays a role in iron transport, but its role in manganese uptake was not demonstrated (4). Using radiolabeled metal, we demonstrated that iron uptake was rapid in the parental W83 strain (Fig. 2A), with the majority of the metal being taken up during

the first 10 min of incubation and then leveling off thereafter. These characteristics are indicative of active uptake. In contrast to the wild type, V2897 (the FeoB1-deficient strain) showed a significantly reduced ability to take up iron, verifying that the protein is a major iron transporter in *P. gingivalis*.

The parental W83 strain also rapidly transported manganese; after 5 min, the majority of the uptake was completed (Fig. 2B). Similar uptake was observed in the FeoB1-deficient strain (V2897), with manganese uptake occurring rapidly and then reaching saturation after 5 min of transport. At 5 min, uptake by the FeoB1-deficient strain was approximately 20% lower than that in the parental strain, but the difference was not statistically significant, and by 20 min, the difference was reduced to 8%.

In order to ascertain that the iron uptake defect was due to the *feoB1* mutation, we also examined the ability of a strain in which the disrupted *feoB1* gene was restored to the parental form. Following 20 min of incubation, while iron uptake by the V2897 strain was reduced 70%, the complemented strain V2979 exhibited iron uptake ability similar to that of the parental W83 strain (Fig. 3). These results demonstrate that the reduced ability to take up iron by V2897 was due to the *feoB1* mutation.

FeoB1 contributes to sensitivity of *P. gingivalis* grown in the presence of oxidative stress. Although iron is an indispensable nutrient for *P. gingivalis*, it can also potentiate oxygen toxicity. As demonstrated above and by others (4), FeoB1 is the major iron transporter and thus contributes to elevated iron levels in the bacterial cell and ultimately may alter the ability of the bacterium to survive in the presence of oxygen. We compared the abilities of each strain to survive exposure to atmospheric oxygen and atmospheric oxygen supplemented with 0.5 mM hydrogen peroxide. No significant viability losses (5%) were observed for either the parental or the mutant strain grown in the presence of atmospheric oxygen (Fig. 4B). However, when cells were incubated in the presence of hydrogen peroxide, significant viability losses were noted (Fig. 4C). It is noteworthy that the FeoB1-deficient strain had a higher survival rate after 180 min of incubation than did the parental strain ($P = 0.02$) (Fig. 4C).

The ability to grow in the presence of peroxide was also examined using a disc diffusion assay (Fig. 5). The extent of the inhibition of microbial growth was dependent on the concentration of peroxide in the discs. Higher peroxide concentrations resulted in larger zones of inhibition for all strains. For the parent, 5% peroxide resulted in a 3.25-mm inhibition zone and 15% peroxide caused a 3.75-mm zone of inhibition. The zones of inhibition for the FeoB1-deficient strain were reduced to 1.35 mm and 2.25 mm, respectively, for the same concentrations of peroxide. This corresponded to 75% and 40% reductions, respectively. The sensitivity was partially restored in the complemented strain V2979. Collectively, these data demonstrate that the presence of the FeoB1 transporter imparts peroxide sensitivity to microbial cells.

FeoB1 contributes to sensitivity of *P. gingivalis* exposed to host cells. *P. gingivalis* attaches to and also survives and multiplies within a variety of host cells. As such, it would be expected to be exposed to oxidative stress generated by the host cells. Indeed, it was demonstrated that host cells do elevate the production of peroxide upon exposure to *P. gingivalis* (8). We hypothesized that reduced iron concentrations associated with the FeoB1 mutation would be beneficial for survival

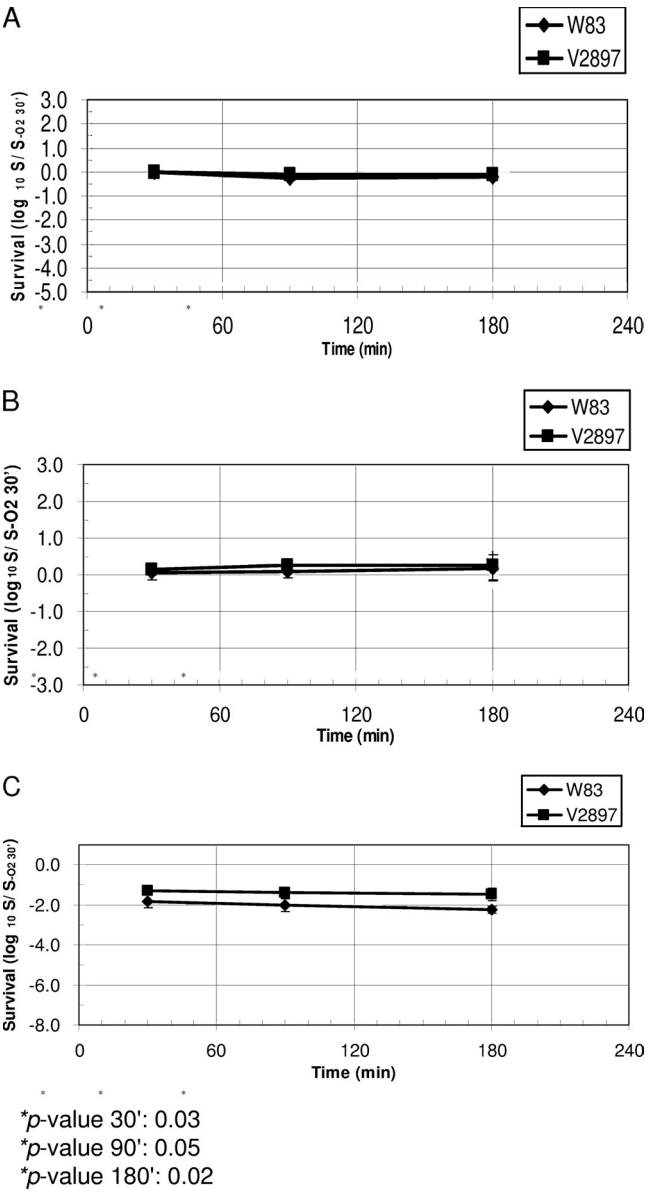

FIG. 4. Sensitivity of *P. gingivalis* strains to oxidative stress. Survival studies were performed over a period ranging from 30 min to 180 min under three experimental conditions: anaerobic conditions (control) (A), atmospheric oxygen (B), and atmospheric oxygen and peroxide (0.5 mM) (C). Survival rates are expressed as $\log_{10}$ ratios of the number of surviving cells in the presence of oxidative stress to the number of cells grown under anaerobic conditions. P values of <0.05 were considered to indicate statistically significant differences (shown by *).

of bacteria in the presence of the oxidative stress generated by host cells. Therefore, *P. gingivalis* strains were exposed to HeLa cells for 30 min, and the total number of bacteria surviving the exposure was tallied (Fig. 6A). The parental strain formed 5,064 CFU/ml, while the FeoB1 mutant strain generated 12,031 CFU/ml (over five times more colonies than the W83 strain). The ability to survive in the presence of host cells was also reduced in the complemented strain, V2979, compared to that in the FeoB1 mutant, V2897. These results demonstrated that the FeoB1 mutant has an increased ability to

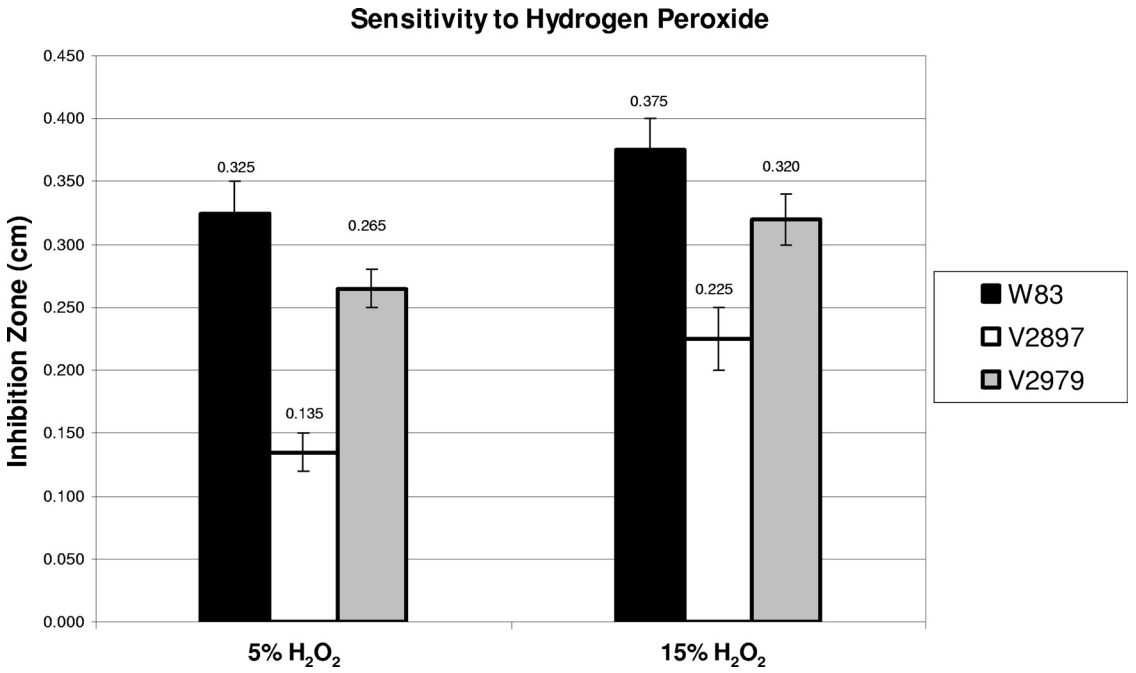

FIG. 5. Peroxide stress sensitivity of *P. gingivalis* strains. Discs saturated with 5% and 15% peroxide were used to examine the ability of peroxide to inhibit microbial growth. Results are means of four measurements.

survive with host cells compared to the parental strain. When extracellular bacteria were killed by the addition of a metronidazole-gentamicin mix and intracellular bacteria were released from host cells and plated, we recovered 1,091 CFU/ml from the parental W83 strain and 4,910 CFU/ml from the FeoB1 mutant strain (Fig. 6B). Reduced numbers of bacteria were also recovered from cells infected with the complemented V2979 strain compared to those infected with V2897, confirming that the fivefold increase in survival rate was due to the FeoB1 mutation (Fig. 6B).

We also examined bacterial invasion rates following 10 min of infection of HeLa cells at an MOI of 10:1, using confocal microscopy. Comparison of the number of viable bacteria recovered from infected cells to the number observed by confocal microscopy showed that only a small fraction of bacteria were able to survive in host cells. While in the case of the parental strain only 0.1 bacterium was able to survive in a single host cell, microscopy analyses showed that as many as 5 bacteria can be internalized by the host cell (data not shown). At the same time, we were able to recover 0.7 bacterium/cell from the FeoB1 mutant, V2897, indicating that this strain has increased survivability when internalized by eukaryotic cells. Furthermore, we observed similar invasion rates for both the parental strain and the mutant strain. At a location 2.0 μm beneath the membranes of HeLa cells, the mean (± standard deviation [SD]) number of bacteria/cell for 20 cells was 5.10 ± 5.59 for the parental strain and 7.30 ± 5.26 for the FeoB1-deficient mutant. Similarly, at the midpoint of the cells, 4.35 ± 4.80 bacteria were observed in cells infected with the parental strain, while the V2897-infected cells contained 6.55 ± 5.34 bacteria. The similar invasion rates demonstrate that the survival differences were not due to differences in the ability of the bacteria to invade cells.

***feoB1* is downregulated upon exposure to host cells.** To gain insight into the mechanisms of regulation of *feoB1* expression, we examined the effects of several conditions on the *feoB1*-specific transcript levels. First, we tested the role of iron on transcript abundance, which logically followed from the function of the transporter. As shown in Table 3, no significant changes in expression level were detected between iron-depleted and iron-replete conditions (the ratio between iron-depleted and iron-replete transcript levels was 0.9). Next, guided by the role of FeoB1 in oxidative stress sensitivity, we examined the impact of hydrogen peroxide on *feoB1* transcript levels. Again, no significant changes were observed following exposure to peroxide. Finally, we investigated the effect of exposure to the host environment on the transcript level. Interestingly, the *feoB1* transcript was dramatically reduced (12-fold) (Table 3) when *P. gingivalis* was grown in conditioned medium. These data demonstrate that *feoB1* expression is regulated by exposure to the host but that the mechanisms involved in that regulation do not require either iron or oxygen.

DISCUSSION

Previous studies showed that the *P. gingivalis* FeoB1 protein is a ferrous iron transporter (4). Here we used a metal uptake assay to show that the protein is required for ferrous iron transport. In addition, we demonstrated that the transporter has no role in high-affinity manganese transport when 0.05 μM manganese is used. However, our unpublished observations showed manganese uptake in the FeoB2-deficient mutant

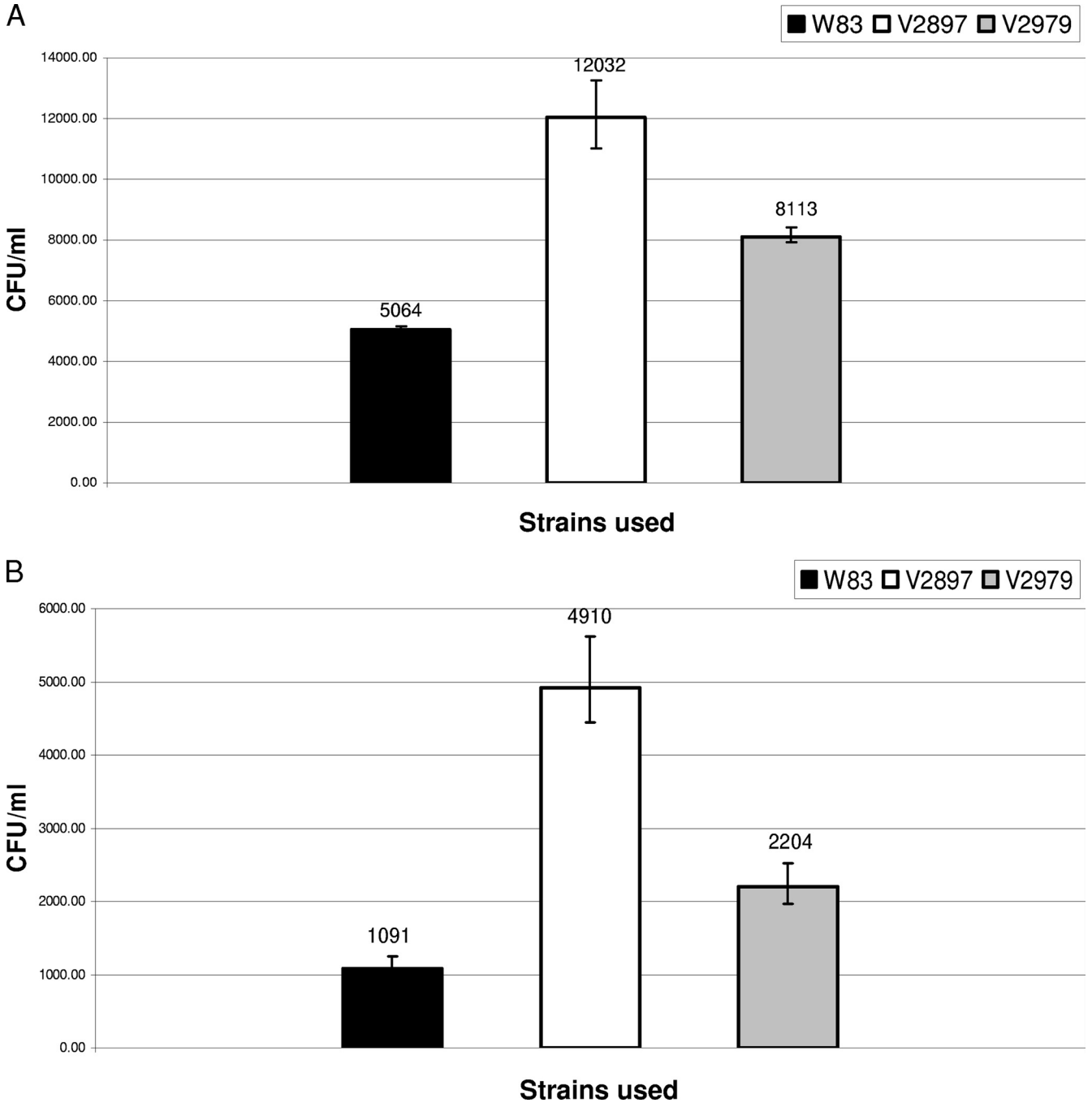

FIG. 6. Survival of *P. gingivalis* in the presence of host cells. *P. gingivalis* parental (W83), FeoB1 mutant (V2897), and complemented (V2979) strains were examined for the ability to survive in the presence of host cells. (A) Total bacteria recovered from HeLa cells. (B) Intracellular bacteria recovered from HeLa cells. Means and standard deviations are from an experiment performed in triplicate.

TABLE 3. qRT-PCR results for *feoB1* expression under various conditions[a]

Presence or absence of growth factor/condition	qRT-PCR result		
	Iron	Hydrogen peroxide	Conditioned medium
Presence	1	1.5	0.08
Absence	0.9	1	1

[a] Results are derived from an experiment performed in triplicate. Control conditions were given a value of 1.

when the bacterial cells were incubated with high concentrations of the metal (>200 nM). These results suggest that in addition to the FeoB2 transporter, another, lower-affinity manganese transporter(s) must be present in *P. gingivalis*. Indeed, given the similarity at the molecular level between the FeoB1 and FeoB2 proteins, as well as the similarity between iron and manganese, it is likely that the FeoB1 protein also transports manganese when it is present at higher concentrations.

We demonstrated that the FeoB1 protein contributes to

oxidative stress sensitivity of *P. gingivalis*. Our results are consistent with another study showing that the deleterious role of iron contributes to oxidative stress generation (18). Our data, however, contradict a previous report that demonstrated that FeoB1 was required for oxidative stress protection in *P. gingivalis* (4). These discrepancies may have resulted from the application of different experimental approaches to examine peroxide sensitivity. One group exposed *P. gingivalis* cells suspended in buffer to peroxide and then examined their survival, while we examined the sensitivity of actively growing cells to peroxide. Since metabolically active bacteria are present in the oral cavity, we believe our results correlate with the *in vivo* scenario.

It is noteworthy that we also showed that the FeoB1 protein contributes to the sensitivity of *P. gingivalis* bacteria exposed to host cells. One of the expected mechanisms playing a role in this sensitivity would be peroxide sensitivity of the microbial cells. Host cells release peroxide, and also, upregulation of peroxide production by cells exposed to *P. gingivalis* has been reported (8). It is probable, however, that other factors also contribute to the elevated sensitivity. Our results are of major biological significance, as we expect that the transporter will decrease the ability of the bacterium to survive in the oral cavity in the presence of oral epithelial cells. Furthermore, *P. gingivalis* has been demonstrated to invade and survive in host cells, and the presence of FeoB1 would be expected to have deleterious effects on survival of the internalized bacterium. This is consistent with previous observations that demonstrated reduced levels of iron uptake proteins in epithelial cell-internalized bacteria compared with those in extracellular *P. gingivalis* (20).

Our results demonstrated that the loss of the function of FeoB1 drastically increased the ability of the bacterium to survive in the presence of host cells, and these findings were confirmed by the substantial downregulation of expression of *feoB1* in conditioned medium observed in our study. It is noteworthy that our expression data are also in agreement with results that demonstrated a reduction in FeoB1 levels in bacteria cultured in gingival epithelial cell-conditioned medium compared to those in *P. gingivalis* grown in control medium (21). This downregulation of potentially toxic proteins seems reasonable. The alteration we observed in the transcription of *feoB1* demonstrated that the regulation of FeoB1 occurs at the transcriptional level. Importantly, iron did not affect *feoB1* expression. This contradicts the results of a previous study (20) that attributed the observed downregulation of metal uptake mechanisms in *P. gingivalis* exposed to host cells to increased iron content of the host cells. Our results demonstrate that the regulation of transcription of *P. gingivalis feoB1* is novel compared with the mechanisms of regulation of previously reported *feoB1* homologs (2). In the latter case, *feoB1* expression is iron dependent and is reduced in iron-replete media. The mechanisms of regulation usually involve the ferric uptake transcriptional regulator Fur (9). Our data show that iron is not the major modulator of expression of this locus and suggest that no iron-dependent regulators play a role in regulation of the locus. These findings promise that novel mechanisms of iron uptake regulation remain to be found in anaerobic bacteria, and therefore, further detailed investigations of the molecular basis of the regulatory mechanisms are warranted.

Given the connection between oxidative stress and iron, we reasoned that exposure to oxygen or peroxide may play a role in regulation of *P. gingivalis feoB1*. Previously, we demonstrated that the expression of a homolog of *feoB1*, the *P. gingivalis* manganese transporter gene *feoB2*, is dramatically elevated upon exposure to oxygen. However, our studies here demonstrated that oxygen does not play a role in regulation of *feoB1*.

In conclusion, because iron uptake may be required for oxidative stress protection and virulence (15), understanding the contribution of the major *P. gingivalis* ferrous iron transporter to these processes is crucial. Our results contradict a previous report on oxidative stress resistance and provide further clarification of the undefined role of iron in the host-pathogen interaction. Iron is an indispensable nutrient for growth of most organisms, and therefore strategies that inhibit metal uptake would be expected to reduce the fitness of the organisms. However, applying such strategies requires caution, because reduced metal uptake may produce adverse effects and inadvertently increase the ability of the microorganism to survive in host cells, which in turn will lead to increased persistence of bacteria in the host.

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