A Novel *Porphyromonas gingivalis* FeoB Plays a Role in Manganese Accumulation*

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FeoB is an atypical transporter that has been shown to exclusively mediate ferrous ion transport in some bacteria. Unusually the genome of the periodontal pathogen *Porphyromonas gingivalis* has two genes (*feoB1* and *feoB2*) encoding FeoB homologs, both of which are expressed in bicistronic operons. Kinetic analysis of ferrous ion transport by *P. gingivalis* W50 revealed the presence of a single, high affinity system with a $K_t$ of 0.31 μM. FeoB1 was found to be solely responsible for this transport as energized cells of the isogenic FeoB1 mutant (W50FB1) did not transport radiolabeled iron, while the isogenic FeoB2 mutant (W50FB2) transported radiolabeled iron at a rate similar to wild type. This was reflected in the iron content of W50FB1 grown in iron excess conditions which was approximately half that of the wild type and W50FB2. The W50FB1 mutant had increased sensitivity to both oxygen and hydrogen peroxide and was avirulent in an animal model of infection whereas W50FB2 exhibited the same virulence as the wild type. Analysis of manganese ion uptake using inductively coupled plasma-mass spectrometry revealed a greater than 3-fold decrease in intracellular manganese accumulation in W50FB2 which was also unable to grow in manganese-limited media. The protein co-expressed with FeoB2 appears to be a novel FeoA-MntR fusion protein that exhibits homology to a manganese-responsive, DNA-binding metalloregulatory protein. These results indicate that FeoB2 is not involved in iron transport but plays a novel role in manganese transport.

The transition metal, iron, is an essential growth requirement for most bacteria due to its redox activity and role in many vital cellular reactions. Iron is a cofactor for many bacterial enzymes involved in redox reactions including oxidoreductases, dehydrogenases, ferridoxins, hydratases, and cytochromes (1, 2). The environmental availability and accumulation of iron play a crucial role in the bacterial pathogenic process. More recently other transition metals, especially manganese, have been shown to play an important role in the control of cellular functions in some bacteria (3). Manganese has also been shown to be essential for resistance to oxidative stress in *Neisseria gonorrhoeae* as it has intrinsic superoxide dismutase activity and can scavenge superoxide (4).

*Porphyromonas gingivalis* has been implicated as a major etiologic agent of chronic periodontitis, an inflammatory disease of the supporting tissues of the teeth (5, 6). *P. gingivalis* is a black-pigmented, anaerobic, asaccharolytic, Gram-negative coccobacillus, which like most bacteria, has an essential growth requirement for iron. The growth and virulence of *P. gingivalis* has been reported to be dependent on the availability of iron complexes such as heme (7–9). Growth studies have indicated that *P. gingivalis* preferentially acquires iron in the form of hemoglobin-derived heme, a molecule comprised of a protoporphyrin IX ring with a co-ordinated central ferrous atom (10). Unlike most bacteria *P. gingivalis* cannot synthesize protoporphyrin IX *de novo* (11, 12) and must also acquire this from the environment, which may explain the preferential utilization of heme by this bacterium. Unlike aerobic or facultative bacteria that obtain iron using siderophores, *P. gingivalis* does not produce siderophores and lacks the ferric reductase activity usually associated with siderophore-mediated iron acquisition (13, 14).

The FeoB family of proteins has been identified from genomic analysis of a number of bacterial species. The predicted FeoB proteins are 700–800 amino acids in length and are integral cytoplasmic membrane proteins that have 7–12 transmembrane-spanning α-helices. The N-terminal region (~300 amino acids in length) is proposed to form a hydrophilic cytoplasmic domain that has one or two nucleotide binding motifs. Few biochemical studies have been conducted to characterize FeoB activity. Both *Escherichia coli* and *Legionella pneumophila* have FeoB proteins that are predicted to be located in the cytoplasmic membrane and have 10 membrane-spanning domains (15, 16). FeoB was shown to be the only ferrous ion transport system in both of these bacteria using isogenic mutants in radiolabeled iron uptake assays. *L. pneumophila* FeoB has 44% identity, 61% similarity to *E. coli* FeoB (16). In addition to FeoB both species express FeoA, a small protein (~75 amino acids in length) of undefined function that is encoded in an operon with *feoB* (15, 16). FeoB has also been reported to be the high affinity iron acquisition system of *Helicobacter pylori*. However there is no FeoA in *H. pylori* and *feoB* is monocistronic (17).

Here we report the discovery of two FeoB-encoding genes (*feoB1* and *feoB2*) in the *P. gingivalis* genome. We have characterized FeoB1 as the ferrous ion transporter, which plays an important role in the virulence of *P. gingivalis* in a murine model of infection. FeoB2 played no role in iron transport but has a novel role in manganese transport.
Novel FeoB Role in Manganese Accumulation

**Experimental Procedures**

**Bacterial Strains and Mutant Production—** *P. gingivalis* strain W50 and the isogenic mutants W50FB1 and W50FB2 were used throughout this study. *P. gingivalis* W50 (ATCC 53978) was obtained from the culture collection of the Center for Oral Health Science, The University of Melbourne. To construct *P. gingivalis* W50FB1 the DNA was amplified from the W50 genome by PCR with oligonucleotide primers that corresponded to nt 183–200 and nt 2498–2472 of the *foeB1* open reading frame, locus PG1294 of the *P. gingivalis* genomic data base (www.tigr.org). The PCR primers included additional EcoRI restriction sites that were used in the ligation of the *foeB1* PCR product into pUCm-T/EcoRI-BAP (Amerham Biosciences, Castle Hill, NSW, Australia). The resulting plasmid pUCfoeB1 was digested with BamHI and ligated with the *erm F-erm AM* cassette, which had been excised from pVA2198 (18) with BamHI/Kpn1, and made blunt using T4 DNA polymerase (New England Biolabs). The resulting pUCfoeB1-erm plasmid was linearized with *AnsI* and transformed into *P. gingivalis* W50. Chromosomal insertional inactivation of *foeB1* in the resulting *erm* spontaneously transformed cells was confirmed by Southern blot analysis.

To construct *P. gingivalis* W50FB2 the *foeB2* gene was amplified from the W50 genome by PCR with oligonucleotide primers that corresponded to nt 318–336 and nt 2140–2123 of the *foeB2* open reading frame (TIGR locus PG1043). The PCR product was digested with *PstI* and transformed into *P. gingivalis* W50. Chromosomal insertional inactivation of *foeB2* in the resulting *Tet* transformants was confirmed by Southern blot analysis. All restriction enzymes were obtained from Promega.

Bacteria were maintained by weekly passaging on horse blood agar plates (40 g/liter blood agar base 2 (Oxoid, Adelaide, SA, Australia), 100 ml/liter horse blood (Taylors Ridge Pty. Ltd., Keysborough, Victoria, Australia)). To maintain the selective pressure, erythromycin (10 μg/ml) or tetracycline (1 μg/ml) was added to the culture medium to maintain the selective pressure.

**Extraction of *P. gingivalis* W50 RNA—** *P. gingivalis* W50 (ATCC 53978) was obtained from the American Type Culture Collection (ATCC). The culture vessel and the medium reservoir were prepared using the mean and standard deviation of multiple assays.

**Growth in Manganese-limited Media—** *P. gingivalis* strains W50, W50FB1, and W50FB2 were initially grown in 20 ml of MBB (BD Biosciences, North Ryde, NSW, Australia) with 0.5 mg/ml cysteine and 5 μg/ml hemin and incubated at 37 °C in an anaerobic chamber for 30 h. A 5% inoculum (v/v) from each starter culture was then added to manganese-limited MBB in a total volume of 250 μl per well in a 96-well microtiter plate (Becton Dickinson). The MBB contained 0.1 M EDTA to MBB. To this 0.5 μg/ml cysteine, 5 μg/ml hemin, 50 μg/ml FeCl₃, 50 μg/ml CaCl₂, 50 μg/ml CuCl₂, 50 μg/ml MgCl₂, 50 μg/ml ZnCl₂, and 5 μg/ml CoCl₂ were added. The plate was incubated at 37 °C in an anaerobic chamber for 30 min, sealed with a plate sealer (PerkinElmer Life Sciences) to maintain anaerobic conditions, and then inserted into a microplate reader that maintained a temperature of 37 °C (Labtech Instruments, Macclesfield, United Kingdom). The growth of the bacteria was monitored every 3 h, with optical density readings recorded at 620 nm (ΔA₆₂₀). Growth curves were constructed using the mean and standard deviation of multiple assays.

**Determination of Cellular Iron Content—** *P. gingivalis* strains W50, W50FB1, and W50FB2 were grown in 100 ml of MBB containing 0.5 μg/ml cysteine, 5 μg/ml hemin, and 5.0 μg/ml heme oxinate. The growth was monitored every 3 h, with optical density readings recorded at 620 nm (ΔA₆₂₀). Growth curves were constructed using the mean and standard deviation of multiple assays.
ml/min aspiration rate with a water jacket-cooled cyclonic spray chamber (both manufactured by Glass Expansion, Victoria, Australia). The sample was freely aspirated into the chamber and was analyzed for the approximate metal content. ICMP was run using multi-element calibration standards (Merck, 5th Granville, NSW, Australia) were dissolved in 5% nitric acid and used prior to sample analysis. Deionized water containing 5% nitric acid was used in between sample aspirations to remove residual ions. The sample aerosol was carried by argon gas directly to the ICP torch (Finnigan quartz torch, 1.5-mm aperture) and was ionized by the Argon plasma. All glassware and plasticware were treated with 35% nitric acid.

Radiolabeled Iron Uptake by P. gingivalis—P. gingivalis W50, W50FB1 and W50FB2 cells grown in batch culture containing 0.5 mg/ml cysteine and 5.0 μg/ml hemin or in continuous culture (see above) were harvested by centrifugation (7500 × g, 20 min, 4 °C). For metabolically killed cells, 10 μM sodium azide was added to each culture, then left to stand 30 min before centrifugation. Each cell pellet was washed in an equal volume of Pbg buffer (10.0 mM NaH2PO4, 10.0 mM KCl, 2.0 mM citric acid, 25 mM MgCl2, 50 μM CaCl2). The cell suspensions in cysteine HCl with the pH adjusted to 7.5 with 5 mM NaOH at 37 °C, resuspended to a cell density of ~3 mg dry weight/ml in the same buffer, and stored on ice until needed. All manipulations were carried out in an anaerobe chamber. Aliquots of the cell suspension (1.2 ml) were warmed to 37 °C, and 12 μl of a bovine serum albumin (BSA) tryptic digest (40 mg/ml) was added to provide a peptide source for the cells 5 min prior to the addition of 24 μl of 55Fe (PerkinElmer Life Sciences, 0.175 MBq/ml; 3.36 MBq/μl). The cell suspensions were then incubated in a final concentration of 1 μM ferric ions. Duplicate samples (0.2 ml) of this cell suspension were centrifuged through silicon oil with a specific gravity of 1.015 g/ml at regular time points to separate the bacterial cells from the buffer as described previously (27). Total and extracellular water volumes of the cell pellet were determined using 1H2O and 13C glucose as described previously (27). Intracellular and extracellular iron concentrations were determined by liquid scintillation counting as described previously (27).

Kinetic analyses of ferrous iron uptake were performed with P. gingivalis W50 cells grown in continuous culture with a ferrous iron concentration range of 0.05–1.00 μM. Ascorbate was added in 100-fold excess to some experiments to reduce any ferric iron to ferrous iron to determine whether ferrous or ferric iron was being transported (16, 17).

Manganese Uptake by P. gingivalis—Manganese uptake by washed cells of P. gingivalis W50 and the FeoB mutants W50FB1 and W50FB2 was determined essentially as described for radiolabeled iron uptake (see above) except that MnCl2 was added to the cell suspension to give a final concentration of 1 μM manganese. Duplicate samples (0.2 ml) of this cell suspension were centrifuged through silicon oil with a specific gravity of 1.015 g/ml at regular time points to separate the bacterial cells from the buffer as described previously (27). Total and extracellular water volumes of the cell pellet were determined using 1H2O and 13C glucose as described previously (27). Intracellular and extracellular iron concentrations were determined by liquid scintillation counting as described previously (27).

Sensitivity to Oxidative Stress—P. gingivalis W50, W50FB1, and W50FB2 cells were harvested by centrifugation during exponential phase growth (8000 × g, 25 min, 4 °C), washed, and suspended in Pbg buffer without cysteine to a cell density of ~109 cfu/ml. The cell suspension was divided into two equal aliquots, and one portion was exposed to H2O2 at a final concentration of 1 mM. The cell suspensions were then incubated aerobically at 37 °C with vigorous shaking for 90 min and sampled at indicated time points. Serial dilutions of both cell suspensions were performed in Pbg buffer containing 0.5 mg/ml cysteine and enumerated by anaerobic culture on horse blood agar plates in quadruplicate. Colonies were counted after 7 days of anaerobic incubation.

Marine Lesion Model—The murine lesion model experiments were approved by the University of Melbourne Ethics Committee for Animal Experiments and conducted essentially as described previously (28). BALB/c mice 6–8 weeks old (10 animals per group) were infected with 2.5–3.0 × 105 viable cells of P. gingivalis strains W50, W50FB1, or W50FB2 by subcutaneous injection (100 μl) in the hind leg, and abdominal lesion size and mortality were monitored over 7 days as described previously (28). The P. gingivalis inocula were prepared from cells grown in batch in BHI broth with 5 μg/ml hemin. Cells were harvested by centrifugation (7500 × g, 20 min, 4 °C) at late exponential phase and washed twice in PG buffer (50 mM Tris-HCl, 150 mM NaCl, 5.0 mM CaCl2, 5 mM cysteine-HCl, pH 8.0). They were then cultured in the anaerobic work station. The number of viable cells in each inoculum was verified by enumeration on horse blood agar. The maximum sizes of the lesions developed were statistically analyzed using the Kruskal-Wallis test and Mann-Whitney U-Wilcoxon rank sum test with a Bonferroni correction for type I error (29).

RESULTS

Identification and Sequence Analysis of Putative P. gingivalis FeoB-encoding Genes—The sequence of the E. coli FeoB protein (15) was used to search the P. gingivalis genome, and two genes were identified whose putative protein products had significant similarity to E. coli FeoB. One gene (feoB1, TIGR locus PG1294) appeared to be monocistronic and was predicted to encode a protein of 844 amino acids that has 38% identity and 65% similarity to E. coli FeoB (Fig. 1). The second gene (feoB2, TIGR locus PG1043) was predicted to encode a protein of 725 amino acids that has 31% identity and 56% similarity to E. coli FeoB. From sequence analysis feoB2 appeared to be in an operon with a gene that is predicted to encode a potential FeoA-DtxR/MntR fusion protein (fea, TIGR locus PG1044) of 310 amino acids that we have designated mntR. P. gingivalis FeoB1 had 31% identity and 57% similarity to FeoB2 (Fig. 1).

Both putative P. gingivalis FeoB proteins were predicted to be integral cytoplasmic membrane proteins with eight (FeoB1) or ten (FeoB2) transmembrane-spanning α-helices in the C-terminal half of the protein and to have large (>300 amino acids) cytoplasmic N-terminal domains. The cytoplasmic N-terminal domain of FeoB proteins has recently been shown to act specifically as a GTPase and not to function as an ATPase as had originally been proposed (30, 31). Both P. gingivalis FeoB1 and FeoB2 contain four of the five characteristic G protein motifs required for GTPase activity that are found in other bacterial FeoBs (Fig. 1; Ref. 30). The N-terminal region of FeoB1 accounts for most of the difference in size with FeoB2. Interestingly the N-terminal 75 amino acids of FeoB1 have 33% identity and 59% similarity to the C-terminal 75 amino acids of P. gingivalis MntR (Fig. 2).

The P. gingivalis MntR C-terminal 75 amino acids have 25% identity (45% similarity) to E. coli FeoA (Fig. 2), while the N-terminal 226 amino acids of the P. gingivalis MntR have 26% identity (48% similarity) to the diphtheria toxin repressor protein (DtxR) of Corynebacterium diphtheriae (32) and 21% identity (48% similarity) to MntR of Bacillus subtilis (33). DtxR and the B. subtilis MntR are DNA-binding metalloregulatory proteins that regulate gene expression in response to iron or manganese, respectively. It has been shown that the amino acid sequence of the primary metal binding site of these proteins plays a major role in defining the metal selectivity of this protein family (34). The primary metal binding site of the iron-responsive DtxR/MntR family of repressors has four essential amino acids, Met10, Cys102, Glu105, and His106 (C. diphtheriae DtxR numbering), whereas the manganese-responsive DtxR/MntR-like repressor binding site has the essential amino acids Asp8, Glu99, Glu102, and His103 (B. subtilis MntR numbering) (34, 35). Interestingly, P. gingivalis MntR exhibits a primary metal binding site of Asp19, Cys108, Glu111, and His112 based on sequence alignments.

FeoB Mutants—To determine the role(s) of the two putative P. gingivalis FeoBs in iron transport, feoB1 (W50FB1) and feoB2 (W50FB2) isogenic mutants were constructed. Chromosomal insertion of ermF-ermAM into feoB1 and tetQ into feoB2 was confirmed by Southern blot analysis (data not shown).

RNA Analysis of FeoB1 and FeoB2—Northern blot analysis indicated that both the feoB1 and feoB2 genes produced a single transcript when P. gingivalis W50 was grown under heme excess conditions (data not shown). The size of the feoB2 transcript (3.4 kbp) was commensurate with feoB2 being transcribed as the second gene in an operon with mntR, as predicted from the DNA sequence, where only 34 bp separate the translational stop codon of mntR and start codon of feoB2. The 3.6-kbp size of the feoB1 transcript, however, was surprisingly
FIG. 1. Alignment of *E. coli* FeoB (EcFeoB; 773 amino acids), *P. gingivalis* FeoB1 (PgFeoB1; 845 amino acids), and *P. gingivalis* FeoB2 (PgFeoB2; 725 amino acids). Identical residues are denoted by a star, conserved residues are denoted by a dot, and dashes denote gaps introduced into the sequence for the purpose of alignment. The four conserved G protein motifs are boxed and labeled.
of peptides and amino acids (36). When energized with the P. gingivalis was used in this study to energize cells in uptake assays, as with excess heme in batch culture transported Fe$_2^+$ less than half that seen in excess heme growth conditions. iron in W50FB1 decreased to 1.4 nmol/mg dry weight as determined by atomic absorp-
tion spectrometry. In contrast, pgFeoB1 Alignment of the P. gingivalis MntR C-terminal 75 amino acids with the P. gingivalis FeoB1 N-terminal 75 amino acids (PgFeoB1) and E. coli FeoA (EcFeoA). Identical residues are denoted by a star, conserved res-
dues are denoted by a dot, and dashes denote gaps introduced into the sequence for the purpose of alignment. Cellular Metal Content—P. gingivalis W50 grown with excess heme had a cellular iron content of 6.1 ± 0.6 nmol/mg cellular dry weight as determined by atomic absorp-
tion spectrometry. In contrast, P. gingivalis W50FB1 had a cellular iron content of 3.4 ± 0.1 nmol/mg dry weight under identical growth conditions (Table I). The effect of insertional inactivation of feoB1 was not, however, restricted to cellular iron content, as manganese levels increased —3-fold relative to the wild type, while zinc and nickel levels decreased. P. gingi-
valis W50FB2 had a similar cellular iron content to the wild type when grown in MBB with excess heme but a two to three times lower intracellular manganese content and a slightly increased zinc level (Table I). When grown in batch culture in MBB under conditions of iron limitation the cellular content of iron in W50FB1 decreased to 1.4 ± 0.3 nmol/mg dry weight, less than half that seen in excess heme growth conditions.

Radiolabeled Iron Uptake—A filtered, tryptic digest of BSA was used in this study to energize cells in uptake assays, as P. gingivalis has been shown to grow on BSA as the only source of peptides and amino acids (36). When energized with the tryptic digest of BSA, washed cells of P. gingivalis W50 grown with excess heme in batch culture transported Fe$_2^+$ at a rate of 16.4 ± 2.9 pmol/mg dry weight/min at an initial extracellular concentration of 1.0 μM (Table II). P. gingivalis W50FB2 grown under identical conditions transported ferrous iron into the cell at a rate of 15.0 ± 6.9 pmol/mg dry weight/min (Table II). In contrast to wild type P. gingivalis, W50FB1 failed to transport radiolabeled iron into the cell. Metabolically killed, washed cells of P. gingivalis W50 did not accumulate Fe$_2^+$ at an extracellular concentration of 1.0 μM. The addition of a 100-fold excess of ascorbate to the incubation had no effect on the rate of uptake.

The growth conditions of the bacterium had a marked effect on the rate of transport of iron into the cell. P. gingivalis W50 grown in continuous culture under heme limitation had twice the rate of iron transport as cells grown with heme excess (Table II). Kinetic analysis of ferrous iron uptake by W50 grown in continuous culture with excess heme revealed the presence of a single system with a $V_{\text{max}}$ of 5.1 pmol/mg dry weight/min and a $K_m$ of 0.31 μM.

Growth in Manganese-limited Media—When grown in batch culture in MBB with excess heme both P. gingivalis and W50 grown in manganese-limited media; however, there was a differ-
ence in both the growth rate and the maximum cell density (0.54 and 0.47 A$_{620\text{ nm}}$ respectively) achieved by these two strains (Fig. 4). W50FB2 did not grow at all under manganese limitation (Fig. 4).

Manganese Uptake—The accumulation of manganese by ener-
ized washed cells of P. gingivalis W50 and the FeoB mutants W50FB1 and W50FB2 was determined using ICP-MS analysis. W50FB2 cellular manganese content was two to three times lower than wild-type W50 (Fig. 5). However, P. gingivalis W50FB1, which already contained a substantially higher cellular content of manganese than W50 or W50FB2, increased its cellular content from 700 to 1164 pmol/mg cellular dry weight in 30 min (Fig. 5).

Sensitivity to Oxidative Stress—When incubated with vigorous shaking in air the viability of P. gingivalis W50 and W50FB2 remained constant for over 90 min (Fig. 6). In contrast, W50FB1 was more sensitive to air with a 50% decrease in viability in 90 min. The addition of hydrogen peroxide caused a 56% decrease in W50 survival in 80 min and had a greater effect on W50FB2 and W50FB1 (Fig. 6). W50FB1 was the most sensitive to hydrogen peroxide exposure with a >70% decrease in viability in 20 min.

Marine Lesion Model—To evaluate the role of FeoB1 and FeoB2 in P. gingivalis virulence, BALB/c mice were infected with the W50FB1 and W50FB2 mutants and the W50 wild type. Lesions that developed were measured and the maximum lesion size recorded. All of the mice infected with 2.5 × 10$^9$ cells of the wild type P. gingivalis W50 developed spreading ulcerative lesions with an average maximum size of 275 ± 82 mm$^2$. Similarly all of the mice infected with W50FB2 developed large spreading ulcerative lesions (321 ± 192 mm$^2$) that were not significantly different in size to those of mice infected with W50 (Fig. 7). In contrast none of the mice infected with 3.0 × 10$^9$ viable cells of the isogenic FeoB1 mutant W50FB1 developed lesions (Fig. 7).

DISCUSSION

The atypical transporter FeoB has been demonstrated to mediate ferrous iron uptake in E. coli, L. pneumophila, and H. pylori (15–17). A BLAST search of the P. gingivalis genome using the E. coli FeoB sequence surprisingly revealed two open reading frames (feoB1 and feoB2) encoding potential FeoB homologs. The two putative P. gingivalis FeoB proteins have a high degree of similarity; the main region of sequence disparity is the N-terminal region of FeoB1 that has significant similarity to E. coli FeoA (Fig. 2) suggesting that P. gingivalis FeoB1 is a FeoA/B fusion protein. RNA analysis showed that not only were the feoB1 and feoB2 genes transcribed under the heme excess growth conditions used in this study, but each gene was also transcribed as part of an operon with a second gene, PG1296 and mntR, respectively. The gene product of PG1296 is a hypothetical protein of 151 residues in length with no significant similarity to known proteins; the lack of an identifiable leader sequence suggests that it is a cytoplasmic protein.

FeoB homologs have not been implicated in the transport of other metals apart from ferrous iron, so from sequence analysis
and of ferrous ion transport by energized cells of bacterium (Table I).

W50 and W50FB2 when grown in media containing excess heme suggested that FeoB1 is involved in iron transport by this strain. Functional FeoB1 (W50FB1) or FeoB2 (W50FB2) were constructed by insertional inactivation of the respective W50 genes. In excess heme both W50FB1 and W50FB2 grew in a similar manner to the wild-type W50 which is consistent with the wild-type W50. In contrast insertional inactivation of feoB1 (W50FB1) abolished the transport of radiolabeled iron, thereby demonstrating the essential role of FeoB1 in ferrous ion transport.

The insertional inactivation of feoB1 led to a 2-fold decrease in P. gingivalis W50 when grown under conditions of heme excess (Table II). The rate of iron uptake was dependent on growth conditions and under heme limitation in continuous culture was more than twice that seen in cells grown in excess heme (Table II). The insertional inactivation of feoB2 (W50FB2) caused no significant difference in the rate of radiolabeled iron uptake by energized P. gingivalis cells relative to the wild type W50. In contrast insertional inactivation of feoB1 (W50FB1) abolished the transport of radiolabeled iron, thereby demonstrating the essential role of FeoB1 in ferrous ion transport.

Despite the presence of two putative FeoBs, kinetic analysis of ferrous ion transport by energized cells of P. gingivalis W50 revealed the presence of a single, high affinity system with a $K_t$ of 0.31 $\mu$m. This is comparable with the only other FeoB transporter that has undergone kinetic analysis, the H. pylori FeoB, which has a $K_t$ of 0.54 $\mu$m (17). The rate of iron uptake was also dependent on growth conditions and under heme limitation in continuous culture was more than twice that seen in cells grown in excess heme (Table II). The insertional inactivation of feoB2 (W50FB2) caused no significant difference in the rate of radiolabeled iron uptake by energized P. gingivalis cells relative to the wild type W50. In contrast insertional inactivation of feoB1 (W50FB1) abolished the transport of radiolabeled iron, thereby demonstrating the essential role of FeoB1 in ferrous ion transport.

The insertional inactivation of feoB1 led to a 2-fold decrease in P. gingivalis W50 when grown under conditions of heme excess. The H. pylori FeoB mutant also had significantly less cellular iron (0.05% dry weight iron) when compared with the wild-type (0.18% dry weight iron; Ref. 17). However, in P. gingivalis W50FB1 a concomitant 3-fold increase in intracellular manganese was also observed. This increase in intracellular manganese content in P. gingivalis W50FB1 may be attributable to the lack of intracellular iron resulting in the up-regulation of another transport system that is capable of transporting manganese into the cell. Interestingly the P. gingivalis genome does not encode an NRAMP family manganese transporter homolog such as MntH. It does, however, encode a putative manganese/iron/zinc-transporting ATP binding cassette transport system (TIGR locus PG1759–1760). The increase in intracellular manganese accumulation may also be the result of manganese ions binding to vacant sites of iron/manganese-binding proteins thus lowering the free manganese ion concentration within the cell driving further uptake.
The insertional inactivation of \textit{P. gingivalis} feoB2, while causing little change in cellular iron levels, led to an almost 3-fold decrease in cellular manganese level (Table I). This indicates that \textit{P. gingivalis} FeoB2 plays a role in the cellular accumulation of manganese. This is the first time that a FeoB transporter homolog has been implicated in the cellular accumulation of non-iron metals and explains the unexpected presence of two FeoB homologs in \textit{P. gingivalis}. The role of FeoB2 in manganese transport is further supported by the \textit{P. gingivalis} \textit{W50FB2} isogenic mutant being unable to grow in a manganese-limited growth medium (Fig. 4). The close linkage between iron and manganese accumulation in \textit{P. gingivalis} is reflected in the unusual cambialistic nature of its superoxide dismutase. Most bacterial superoxide dismutases specifically utilize either iron or manganese as the metal cofactor to catalyze the disproportionation of the superoxide radical into hydrogen peroxide and molecular oxygen. However, the superoxide dismutases of a few bacteria including \textit{P. gingivalis} and \textit{Bacteroides fragilis} can utilize either manganese or iron to give maximum specific activity (37, 38). This flexibility in superoxide dismutase metal ion specificity may have evolved to aid \textit{P. gingivalis} exploit habitats where iron is not freely available and may have resulted in a more coordinated balance between iron and manganese cellular content than seen in other bacteria.

\textit{P. gingivalis} can be isolated from inflamed periodontal pockets where the subjacent tissue contains an infiltrate of polymorphonuclear leukocytes. Polymorphonuclear leukocytes produce superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) as part of their bactericidal armory. These reactive oxygen species and the hydroxyl radical (OH) can cause damage to DNA, proteins, and lipids. Intracellular manganese has been shown to protect against the deleterious effects of reactive oxygen species.
species in a number of aerobic and aerotolerant bacteria (4, 39). No analysis of anaerobic bacteria has been undertaken to determine whether they can be protected from oxidative stress by intracellular manganese accumulation. In light of this we tested the ability of the FeoB mutants to survive in the presence of air and hydrogen peroxide as they have altered levels of cellular manganese and iron (Table I). W50FB1, which has by far the highest manganese content, proved to be more sensitive to both the presence of air and hydrogen peroxide than the other strains (Fig. 6). This indicates that in P. gingivalis cellular iron content is more important than intracellular manganese for protection against oxidative stress as W50FB1 has the lowest cellular iron levels.

In P. gingivalis, FeoB2 is encoded in an operon with another gene that encodes a novel protein, designated MntR, that appears to be a FeOA-DtxR/MntR fusion protein. The P. gingivalis MntR C-terminal 75-amino acid sequence has similarity to E. coli FeoA (Fig. 2), while the N-terminal 226-amino acid domain of P. gingivalis MntR has extensive similarity to DtxR of C. diptheriae and MntR of B. subtilis, both of which are members of the DtxR/MntR family of global metalloregulatory proteins (34, 35, 40). Although the P. gingivalis MntR has a metal binding site that contains elements of both the proposed iron-specific and manganese-specific binding sites (34), we propose that MntR is involved in regulation of manganese rather than iron transport within P. gingivalis. It is unlikely that the P. gingivalis MntR is an iron-responsive DtxR homolog given that such proteins are usually found in the high GC branch of Gram-positive bacteria (41). In contrast Fur is the iron-responsive regulator found in Gram-negative bacteria and in Gram-positive bacteria with low GC content (41). A gene predicted to encode a Fur homolog has been identified in the E. coli MntR suggests an interaction between FeoB2 and MntR, as both of which are positive regulators found in Gram-negative bacteria and in Gram-positive bacteria (41). In contrast Fur is the iron-responsive regulator found in Gram-negative bacteria and in Gram-positive bacteria with low GC content (41). A gene predicted to encode a Fur homolog has been identified in the E. coli MntR suggests an interaction between FeoB2 and MntR, as both of which are positive regulators found in Gram-negative bacteria and in Gram-positive bacteria (41).

The role of ferrous iron transport in the virulence of P. gingivalis was determined using an established mouse lesion model of infection (28). Animal model data showed that inserntional inactivation of feoB1 rendered the bacterium avirulent when tested in this murine model. No mice infected with W50FB1 developed any lesions, in contrast to mice infected with W50FB2 that developed lesions that were not significantly different in size to those infected with the wild-type W50. The avirulence of W50FB1 correlates with the increased sensitivity of this strain to oxidative stress. The inactivation of FeoB in H. pylori rendered the mutant unable to colonize the gastric mucosa of mice (17). An E. coli FeoB mutant was also unable to colonize the mouse intestine, and a L. pneumophila FeoB mutant was attenuated for intracellular growth (16, 42). Together these data demonstrate the crucial role of FeoB-mediated ferrous iron transport in the virulence of a variety of bacteria.

In conclusion, we have demonstrated that P. gingivalis uniquely expresses two FeoB proteins. Ferrous iron transport is mediated by FeoB1, and this iron transport is essential for the virulence of the bacterium. FeoB2 in concert with a novel FeOA-MntR fusion protein mediates manganese transport.