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The Divergently Transcribed *Streptococcus parasanguis* Virulence-Associated *fimA* Operon Encoding an Mn^{2+}-Responsive Metal Transporter and *pepO* Encoding a Zinc Metallopeptidase Are Not Coordinately Regulated

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The study of how bacteria respond to and obtain divalent metal ions provides insight into the regulation of virulence factors in the host environment. Regulation of metal permease operons in gram-positive bacteria may involve the binding of metal-responsive repressors to palindromic domains in their control regions. The *Streptococcus parasanguis fimA* operon, which encodes an ATP-binding cassette (ABC) transporter system with sequence homology to the LraI family of metal transporters, possesses a palindromic regulatory region with high homology to that of the *Streptococcus gordonii* ScaR binding domain. Mapping of the promoter and regulatory regions of *fimA* and the divergently transcribed *pepO* gene, which encodes a zinc metalloendopeptidase, indicated that their promoter and regulatory elements overlap. *fimA* had one transcriptional start site, whereas *pepO* had three. Analysis of truncated versions of the *pepO* promoter suggested that all three transcriptional start sites are functional. Analysis of promoter activity under various environmental conditions indicated that the *fimA* operon promoter and the *pepO* promoter are not coordinately regulated. The *fimA* operon is responsive to changes in Mn^{2+} concentration, but the *pepO* promoter is not. A *S. parasanguis fimA* mutant showed a growth deficiency under conditions of limiting Mn^{2+}. This deficiency was not alleviated by compensation with either Mg^{2+} or Fe^{3+}. Wild-type *S. parasanguis* could take up Mn^{2+} and Fe^{3+}, while the *fimA* mutant showed a marked reduction in this ability. These data suggested that FimA is a component of a metal transporter system capable of transporting both Mn^{2+} and Fe^{3+}. FimA expression itself was shown to be responsive to Mn^{2+} concentration, but not to availability of Fe^{3+} or Mg^{2+}.

Streptococcus parasanguis, along with other members of the mitis group of oral streptococci, are among some of the most successful colonizers of the human body. These commensal organisms in the oral cavity have the ability to attach, colonize, and thrive in an environment of continual flux of pH, temperature, mechanical stress, and nutrient availability. Introduction of these oral organisms into the bloodstream of individuals with predisposing heart valve damage can result in endocarditis, a life-threatening illness (3). Nutrients, in particular divalent metal ions, are often sequestered by the host in such a way that the colonizing bacteria must actively gain access to these resources in order to survive in the host environment. Iron in the form of ferric and ferrous compounds is essential for the growth and survival of gram-negative bacterial pathogens, and the ability to acquire these nutrients is considered a virulence trait (8, 34). In gram-positive organisms, such as the streptococci, the role of divalent metals in virulence is less well-defined. Evidence indicates that the lipoprotein receptor-associated antigen I (LraI) family of polypeptides found in a variety of streptococci (4, 7, 22, 23, 35) and of which FimA of *S. parasanguis* FW213 is a member forms part of a new family of solute-binding receptors of ABC metal ion transporters. Previous studies have shown that *Streptococcus gordonii* (24) and *Streptococcus pneumoniae* (9) transporters mediate uptake of Mn^{2+}, while recent work indicates that the *Streptococcus pyogenes* LraI polypeptide binds Zn^{2+}, Cu^{2+}, and Fe^{3+} (21).

S. parasanguis FimA is a major virulence factor associated with endocarditis, and it has been suggested that FimA functions in the development of the infection by facilitating adherence to fibrin (5). Other members of the LraI family, including PsaA of *S. pneumoniae* and SloC of *Streptococcus mutans*, have also been shown to be virulence factors in animal models (4, 23). A potential virulence factor in *S. parasanguis*-induced endocarditis may be the zinc metallopeptidase PepO, which has been shown to be highly homologous in sequence and activity to the vasoconstriction-associated mammalian endothelin-converting enzyme (14, 28). The divergently transcribed *pepO* gene is immediately upstream of the *fimA* operon. It has been suggested that the promoter domains of the *fimA* operon and *pepO* gene overlap and that the two may be coordinately regulated (14). Regulation of transcription is often the means by which metal ion acquisition is regulated (15, 20, 33). Classic examples of this are the ferric uptake repressor (Fur) of *Escherichia coli* and the diphtheria toxin repressor (DtxR) of *Corynebacterium diphtheriae*. These metal-responsive repressors bind to regulatory regions controlling the transcription of genes involved in metal transport, siderophore production, and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. parasanguis		
FW213	Wild-type	6
VT930	FW213 *fimA*::*aphA3*	13
VT1393	FW213 *fapI*::*aphA3*	37
VT1548	FW213 carrying pVT1550	This study
VT1549	FW213 carrying pVT1551	This study
VT1553	FW213 carrying pVT1552; control for luciferase assay	This study
E. coli HB101	Host strain for cloning and plasmid propagation	Promega
Plasmids		
pT7Blue	2.9-kb PCR cloning vector; Ap^r	Novagen
pUC18	2.7-kb cloning vector; Ap^r	New England Biolabs
pVT1198	pUC18 containing portions of *pepO* and *fimC;* Ap^r	14
pVT1327	pT7Blue containing portions of *pepO* and *fimC;* Ap^r	14
pSG223	*E. coli*-streptococcal luciferase shuttle vector; luciferase gene under the control of the *gtf* promoter; Er^r	16
pVT1550	pSG223 with the *pepO* promoter replacing the *gtf* promoter 5′ of the luciferase gene; Er^r	This study
pVT1551	pSG223 with the *fimA* operon promoter replacing the *gtf* promoter 5′ of the luciferase gene; Er^r	This study
pVT1552	Promoterless pSG223; control for luciferase assay; Er^r	This study

expression of virulence factors (11, 19). Recently, it has been shown that *S. gordonii* possesses a metallorepressor, ScaR, that shares 26% identity with DtxR of *C. diphtheriae*. This repressor, under conditions of high Mn^{2+} concentration, binds a regulatory region in the promoter of the *S. gordonii fimA* operon homolog, *sca*, thereby repressing transcription (20). The regulatory domain to which ScaR binds appears to have high DNA sequence homology to palindromic domains in the promoter regions of genes encoding other LraI family members, including the ABC-type transporter of *S. parasanguis*.

Studies were undertaken to examine the promoter activity of the *fimA* operon and *pepO* gene under conditions of limiting divalent metal ion availability. We show here that the *S. parasanguis fimA* promoter is repressed under conditions of high Mn^{2+} but that the divergently transcribed *pepO* promoter is not. In this paper, we have also characterized the function of FimA further and now have several lines of evidence which indicate that FimA is a metal transporter that is responsive to Mn^{2+} concentration.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. The wild-type *S. parasanguis* (formerly called *S. sanguis*) strain used was FW213 (6). VT930 and VT1393 are isogenic derivatives of FW213. Streptococcal strains were grown statically in the presence of 5% CO_2 at 37°C in Todd-Hewitt (TH) (Difco Laboratories, Detroit, Mich.) broth or in a chemically defined medium (CDM) (JRH Biosciences, Denver, Pa.). For analysis of the effects of metals on cell growth, CDM was prepared and incubated with 30 g of Chelex-100 (Sigma) per liter for 4 h at 4°C to remove trace metals (32). The medium was filter sterilized, and metal ions were restored by the addition of $MgSO_4$ 52 $7H_2O$, $MnCl_2{\cdot}4H_2O$, or $FeCl_3{\cdot}6H_2O$ as described below. Growth in CDM or TH broth was monitored by measuring the optical density at 600 nm (OD_{600}) or 470 nm (OD_{470}) (Spectronic 20D; Milton Roy Company, Rochester, N.Y.), respectively, or by enumerating CFU. VT1548, VT1549, VT1553, and FW213 strains containing the *E. coli*-streptococcal luciferase shuttle vectors were grown in medium containing 10 μg of erythromycin per ml for plasmid selection.

E. coli strain HB101 was grown at 37°C in Luria-Bertani (LB) broth or LB agar plates with the addition of 500 μg of erythromycin per ml to select for the presence of the *E. coli*-streptococcal luciferase shuttle plasmid encoding an erythromycin resistance marker.

Preparation of whole-cell extracts. Streptococcal cultures were grown under specific environmental conditions to early logarithmic growth phase (OD_{470} of 0.3 or OD_{600} of 0.45) and mid-logarithmic growth phase (OD_{470} of 0.6 or OD_{600} of 0.9). Whole-cell extracts were prepared as previously described (28) except that cell pellets were resuspended in 20 mM potassium phosphate buffer for immunoblot analyses or 1× reporter lysis buffer (RLB) (Promega Corporation, Madison, Wis.) for luciferase assays. Protein concentrations were determined by using the bicinchoninic acid protein assay reagent kit (Pierce Chemical Company, Rockford, Ill.) with bovine serum albumin as the standard.

Primer extension analysis. Total RNA was obtained from FW213 cells grown in TH broth to late log growth phase (OD_{470} of 0.8). Cells were pelleted by centrifugation and processed using the Bio 101 FastRNA Kit (blue) and the FP120 FastPrep machine (Bio 101, Inc., Vista, Calif.) according to the manufacturer's recommendation. Primer extension analysis was performed as described previously (36). The oligonucleotide 5′-CCCTGGATGGTGAGGGAAAG-3′ was used to map the start of transcription of the *fimA* operon by primer extension. The oligonucleotide 5′-GTAAAGGAGCAAACTCATG-3′ was used to map the start of transcription of the first *pepO* transcript, and the oligonucleotide 5′-CATTCCCCGTTGACGTAATC-3′) was used to map the start of transcription of the second and third *pepO* transcripts and to confirm the transcriptional start site of the first *pepO* transcript. Oligonucleotide primers were 5′ end labeled using T4 polynucleotide kinase and γ-^{32}P-labeled dATP. The labeled oligonucleotide was hybridized with 10 μg of *S. parasanguis* total RNA, and extension was performed using SuperScript RNase H^- reverse transcriptase (Gibco BRL) for 1 h at 42°C. The extended product was denatured and loaded onto a 6% polyacrylamide gel which contained 7 M urea. DNA sequencing (Sequenase version 2.0, DNA Sequencing Kit; U.S. Biochemicals, Cleveland, Ohio) of pVT1198 or pVT1327 containing the relevant portions of the *fimA* and *pepO* loci were performed using the same oligonucleotides as primers, and the products of these reactions were used as molecular size markers for the primer extensions.

Construction of the *fimA* operon and *pepO* promoter-luciferase fusion plasmids. The wild-type *pepO* and *fimA* operon promoters from pVT1327 were amplified by PCR using a Techne Genius thermocycler (Techne Ltd., Cambridge, United Kingdom) and the GeneAmp PCR reagent kit (Roche Molecular Systems, Inc., Branchburg, N.J.). As can be seen in Fig. 2B, oligonucleotide primers designated *pepO* primer 5′ (5′-GGA**AGATCT**TTTTGATCAAGCTGG-3′) and *pepO* primer 3′ (5′-GGT**CCATGG**ATCTTCTCGCTTTCATTC-3′) were designed to amplify regions of the *pepO* promoter, and *fim* primer 5′ (5′-CCT**AGATCT**TGCTGGTATAGTCTTC-3′) and *fim* primer 3′ (5′-GGT**CCATGG**AGTTTGCTCCTTTAC-3′) were designed to amplify regions of the *fimA*

operon promoter. The primers were also constructed to contain *Bgl*II or *Nco*I restriction sites (shown in bold type) to facilitate cloning of the amplified promoter regions into the respective sites in the *E. coli*-streptococcal luciferase shuttle plasmid pSG223, thereby replacing the *gtf* promoter of pSG223 with the *fimA* operon or *pepO* promoter and generating pVT1550 or pVT1551, respectively. Plasmids were electroporated into electrocompetent *E. coli* HB101 cells following previously described protocols (2) using a Gene Pulse apparatus (Bio-Rad Laboratories, Hercules, Calif.). Cloned plasmids were isolated from *E. coli* using minicolumn plasmid purification kits (Qiagen, Inc., Santa Clarita, Calif.). DNA sequence analyses of the plasmids were performed at the Vermont Cancer Center DNA Analysis Facility at the University of Vermont by the Sanger dideoxynucleotide chain termination method as modified for ABI Prism Dye Terminator cycle sequencing using ampliTaq polymerase on an ABI 373 XL automated DNA sequencer (Perkin-Elmer Cetus, Norwalk, Conn.). Sequencing indicated that the *pepO* and *fimA* operon promoters were in frame and in proper orientation for the production of the luciferase protein. The *E. coli*-streptococcal reporter plasmids were transformed into FW213 cells by electroporation (12). The transformants VT1548 and VT1549 were used in luciferase assays to measure *pepO* or *fimA* operon promoter activity, respectively.

Luciferase assay. Luciferase assays were performed as recommended by the manufacturer of the Luciferase Assay System Kit (Promega Corporation) containing RLB with the following modifications. Bacterial cells were grown under specific environmental conditions, and whole-cell extracts were prepared. Total protein (1 μg/ml) from whole-cell extracts was used for each assay in a total volume of 20 μl of 1× RLB. Assayed samples were equilibrated to room temperature, 100 μl of luciferase assay reagent (Promega Corporation) was added to each sample prior to analysis, and relative light units were determined in a LB9501 Berthold Lumat luminometer (Perkin-Elmer Berthold, Wellesley, Mass.).

Metal uptake and competition. Cells grown in TH broth to mid-logarithmic phase were diluted in fresh TH broth to approximately 10^6 cells ml^{-1} (OD_{470} of 0.01). For metal uptake experiments, 2.0 μCi of either ^{54}Mn (0.8 μM) (Dupont NEN, Boston, Mass.) or ^{55}Fe (4.0 μM; Dupont NEN) was added to 1.0 ml of culture. Cultures without labeled metals were included to determine the numbers of CFU at the end of the experiment. Bacteria were grown overnight at 37°C and 5% CO_2 and washed three times with fresh medium. Cells grown with ^{54}Mn were washed in fresh medium containing 150 μM $MnCl_2$ 52> $4H_2O$; those grown with ^{55}Fe were washed with 300 μM $FeCl_3 \cdot 6H_2O$. After the final washes, cell pellets were resuspended in 100 μl of TH broth, transferred to 5 ml of Cytoscint scintillation fluid (ICN Biomedicals, Inc., Costa Mesa, Calif.), and radioactivity was measured in a Beckman LS6500 scintillation counter using a window calibrated for the appropriate isotope. Competition assays were performed as described above except that excess unlabeled Mn^{2+} or Fe^{3+}, as indicated, was incubated with the cells along with radiolabeled Mn^{2+} or Fe^{3+} in order to evaluate the specificity of the binding interaction.

Immunoblot analysis of FimA and PepO. *S. parasanguis* cultures were grown in Chelex-100-treated CDM supplemented with appropriate metal ions to the late logarithmic growth phase (OD_{600} of 1.2). Cells from 1-ml aliquots were harvested by centrifugation and resuspended in 100 μl of sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol). For immunoblot analysis using whole-cell extracts, 1 μg of total protein was added to sample buffer. Samples were boiled for 10 min, centrifuged, and separated by electrophoresis on 12% polyacrylamide–SDS gels. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, N.H.) and examined by immunoblot analysis (33a) as described previously (37) except that a 1:5,000 dilution of PepO antiserum was used as a probe for PepO and a 1:2,500 dilution of FimA antiserum was used for FimA. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G at a dilution of 1:10,000 was used as the secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.). Antibody conjugates were detected with a chemiluminescence system as described by the manufacturer (NEN Life Science Products, Boston, Mass.).

RESULTS

The promoter and regulatory regions of *S. parasanguis fimA* and *pepO* overlap. Previous analysis indicated that *pepO* was transcribed as a monocistronic message with at least two transcriptional start sites, as evidenced by a major and minor transcriptional product in Northern blot analysis (14). Primer extension analysis was performed to further elucidate the exact number of *pepO* transcriptional start sites and to investigate the potential overlap between the *pepO* and *fimA* operon promoters. The analysis indicated that *pepO* transcription was initiated at three transcriptional start sites producing three transcriptional products (Fig. 1B and C). The first transcriptional start site occurred 267 nucleotides from the start of translation, whereas the second and third sites were 155 and 123 nucleotides from the ATG start site, respectively. The two shorter transcription products were separated by 32 nucleotides and thus were not resolved on the previous Northern hybridization analysis. The first *pepO* transcription product occurred 118 nucleotides into the *fimA* coding region and 134 nucleotides from the *fimA* start of transcription (Fig. 1A), indicating that the promoter regions of *pepO* and the *fimA* operon overlap. Using the information provided by primer extension analysis, a schematic diagram indicating the putative −10 and −35 regions of the *fimA* operon and *pepO* promoters was constructed (Fig. 2).

The *fimA* operon promoter is responsive to the Mn^{2+} concentration, but the *pepO* promoter is not. The organization of the *fimA* operon and the divergently transcribed *pepO* was described previously (14). A similar gene arrangement is seen with the *S. gordonii sca* operon and its divergently transcribed *pepO* (20, 25). On the basis of DNA sequence alignment and homology, it was suggested that the metallorepressor binding region that exists in the promoter of the *sca* operon of *S. gordonii* may also be present in the *fimA* operon of *S. parasanguis* (20). It was determined (Fig. 3) that the promoter domain of *S. parasanguis pepO* overlaps the promoter domain of the *fimA* operon and also includes a potential binding region for a metalloresponsive repressor. This suggests that transcription of both the *fimA* operon and *pepO* gene may be responsive to metal availability, since key transcriptional elements for both the *fimA* operon and *pepO* promoters would potentially be sequestered by the repressor. This hypothesis was investigated by using *E. coli*-streptococcal luciferase reporter shuttle plasmids pVT1551 and pVT1550. The *gtf* promoter of pSG223 was replaced by regions of the *fimA* operon promoter (Fig. 2), 463 nucleotides 5′ of the *fimC* start of translation, or the *pepO* promoter (Fig. 2), 335 nucleotides 5′ of the *pepO* translational start site, thereby placing the luciferase gene under the control of the *fimA* operon or *pepO* promoters, respectively. Luciferase assays of whole-cell extracts of *S. parasanguis* strains VT1548, VT1549, and VT1553 (control) grown to various growth phases in the presence of 0.1 or 10 μM Mn^{2+} indicated that the *fimA* operon promoter activity was repressed as much as threefold if grown in the presence of 10 μM Mn^{2+} (Fig. 4A). The promoter activity of *pepO*, however, was unresponsive to changes in Mn^{2+} concentration (Fig. 4B). The luciferase activity of both promoter constructs increased dramatically in mid-logarithmic growth phase compared to that of early logarithmic growth phase, suggesting that promoter activity of the *fimA* operon and *pepO* promoters may be regulated by growth phase. Two other promoter constructs were generated to investigate the possibility that the promoter regions for the two smaller *pepO* transcripts, the second and third *pepO* transcripts, may be responsive to Mn^{2+} concentration. These *pepO* promoter constructs were also unresponsive to Mn^{2+}, but each did support transcription of the luciferase gene, indicating that each of these domains acts as a functional promoter (data not shown). The *pepO* promoter constructs also showed luciferase activity at least 10,000 times greater than that of the *fimA*

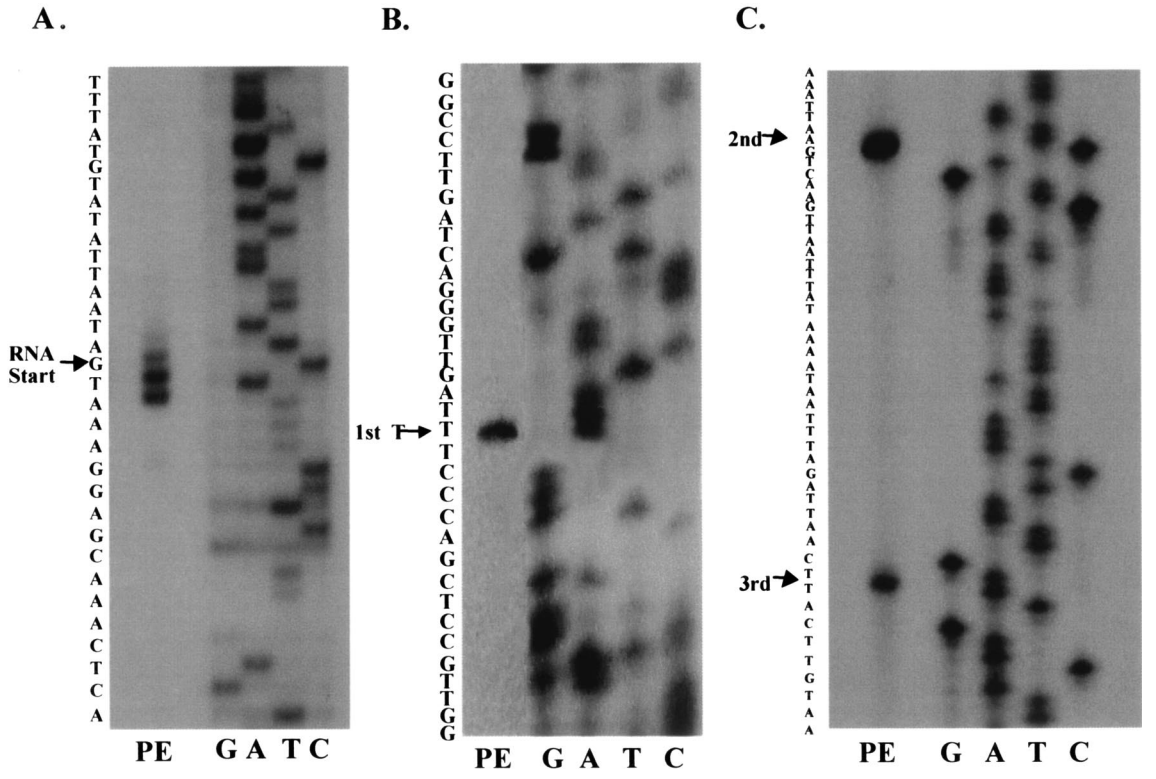

FIG. 1. Primer extension (PE) analysis of the *fimA* (A) and *pepO* (B and C) transcripts. In lane PE, oligonucleotide was annealed to 10 μg of *S. parasanguis* FW213 RNA and extended using SuperScript RNase H⁻ reverse transcriptase. The nucleotide sequences of pVT1198 and pVT1327 (lanes G, A, T, and C) were determined using the same oligonucleotide as a primer.

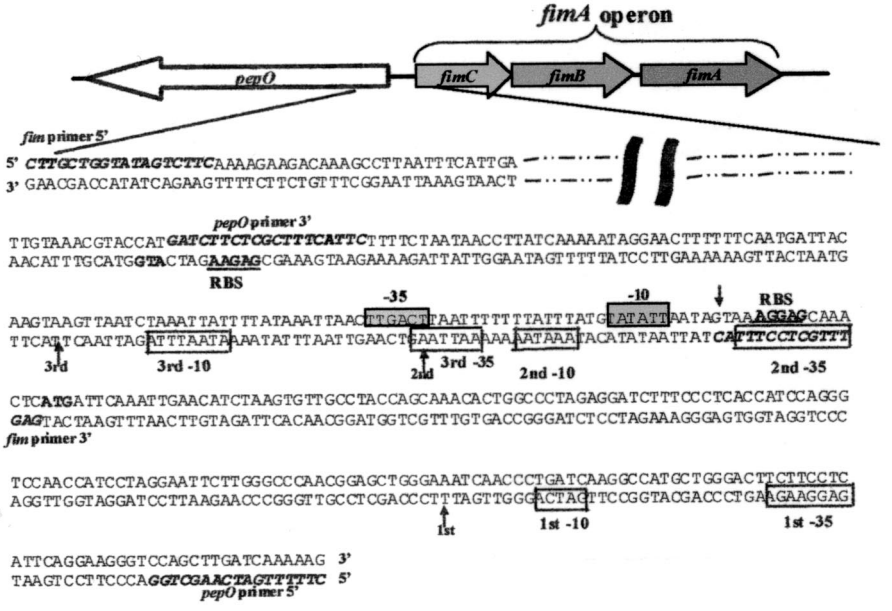

FIG. 2. Schematic representation of the orientation of the *pepO* gene and the *fimA* operon in *S. parasanguis*. The expanded region shows the nucleotide sequence encompassing the 5′ coding region of *pepO*, the intergenic region between *pepO* and the *fimA* operon, and the 5′ coding region of the *fimA* operon. The *fimA* operon sequence (top sequence) and *pepO* sequence (bottom sequence) are shown. Sequence reported elsewhere (12) (dashed and dotted line) and sequences to which designated primers were designed for PCR and cloning purposes (bold italic type) are indicated. The arrows indicate the location of the start(s) of transcription for either the *fimA* operon or *pepO* gene, as determined by primer extension analysis. For the genes of the *fimA* operon and *pepO* gene, the start site of translation (bold type) and putative ribosomal binding sites (RBS) (underlined sequence) are shown. The putative −10 and −35 sites of the *fimA* operon (boxed sequence on grey background) and the *pepO* promoter (boxed sequence on white background) are indicated.

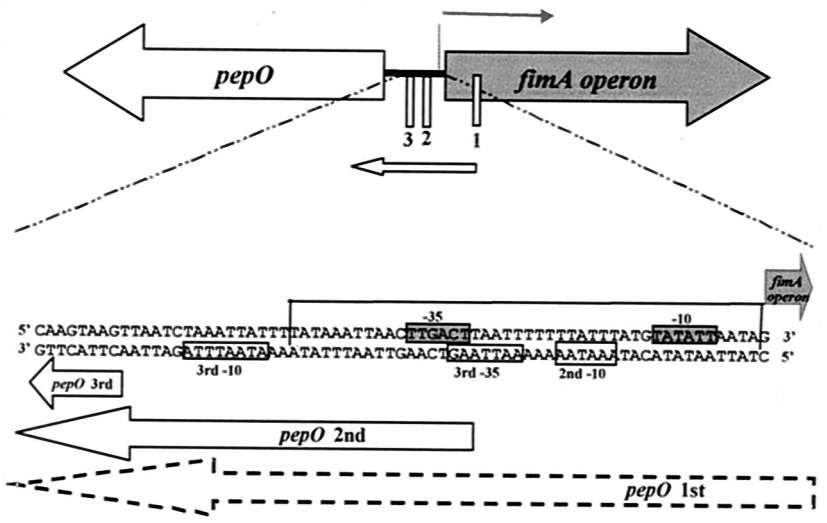

FIG. 3. Schematic diagram of the *S. parasanguis pepO* gene and the *fimA* operon. The start of transcription of the *fimA* operon (thick black line) and the three start sites of *pepO* transcription (thin white boxes) are indicated. The expanded region shows the area of overlap between the *fimA* operon promoter and the *pepO* promoter. The *fimA* operon sequence (top sequence) and *pepO* sequence (bottom sequence) in the designated 5′ to 3′ orientations are shown. The region that shares high DNA sequence identity with the metallorepressor binding domain in *S. gordonii sca* operon (20) is boxed on three sides. The putative −10 and −35 sites of the *fimA* operon (boxed sequence on grey background) and the *pepO* promoter (boxed sequence on white background) are indicated. The arrows represent transcription products from either the *fimA* operon promoter or the *pepO* promoter.

operon promoter, indicating that the overall strength of the *pepO* promoter is much greater than that of the *fimA* operon promoter (Fig. 4).

***S. parasanguis* displays a FimA-dependent growth requirement for Mn^{2+}.** Since the *fimA* operon promoter of *S. parasanguis* FW213 was responsive to Mn^{2+} concentration, further investigation of the effects of divalent metal ions on bacterial growth was investigated. Growth of the *fimA* mutant strain VT930 in CDM containing low levels of Mn^{2+} (0.01 to 0.1 μM) was severely inhibited compared with that of wild-type FW213 (Fig. 5A). Growth of the *fimA* mutant began to be restored when the medium was supplemented with additional Mn^{2+} (>0.1 μM), and the OD_{600} of the culture approached that of the wild-type strain when >5.0 μM Mn^{2+} was added. Variation in the concentration of other ions, such as Mg^{2+} (Fig. 5B) and Fe^{3+} (data not shown), did not produce any further

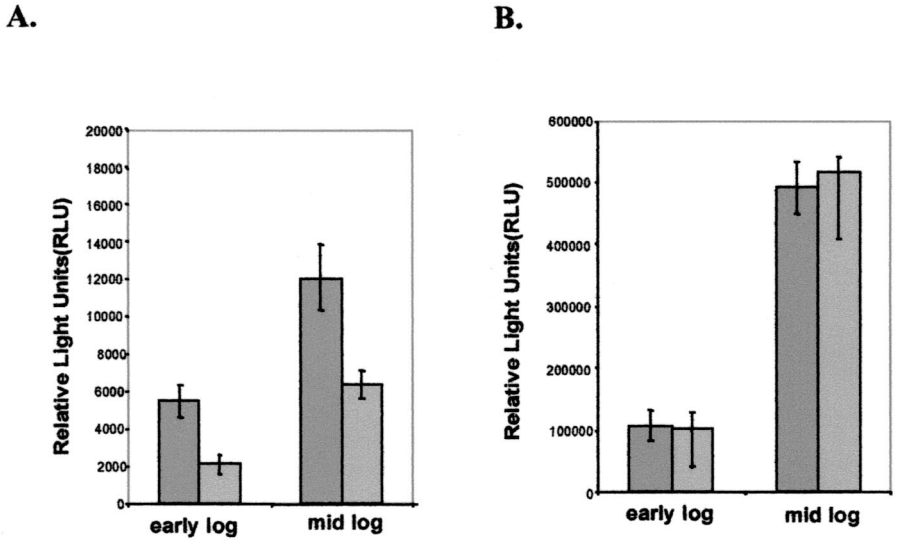

FIG. 4. Luciferase assay of VT1549 and FW213 strains containing either the *fimA* operon promoter construct (A) or the *pepO* promoter construct (B), grown in Chelex-100-treated CDM supplemented with 5.0 μM Mg^{2+} and 0.1 μM Fe^{3+} and various concentrations of Mn^{2+} (0.1 μM [dark grey bars] or 10 μM [light grey bars]). One microgram of total protein of whole-cell extracts in a final volume of 20 μl of RLB was mixed with 100 μl of luciferase assay reagent, and the light produced was measured in a luminometer. These experiments were performed four times; means and standard errors (error bars) are shown and adjusted for controls.

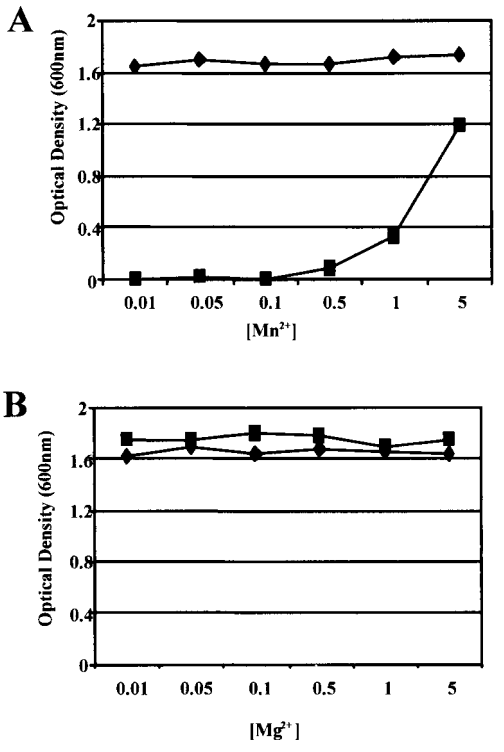

FIG. 5. Effects of Mg^{2+} and Mn^{2+} concentrations on growth of wild-type FW213 and *fimA* mutant. Wild-type FW213 (♦) and *fimA* mutant (■) were grown in the presence of 1.0 μM Mg^{2+} and various concentrations of Mn^{2+} (A) and 1.0 μM Mn^{2+} and various concentrations of Mg^{2+} (B). Cells were grown for 16 h in Chelex-100-treated CDM supplemented with 1.0 μM Fe^{3+} and metals as indicated, and cell density (OD_{600}) was measured.

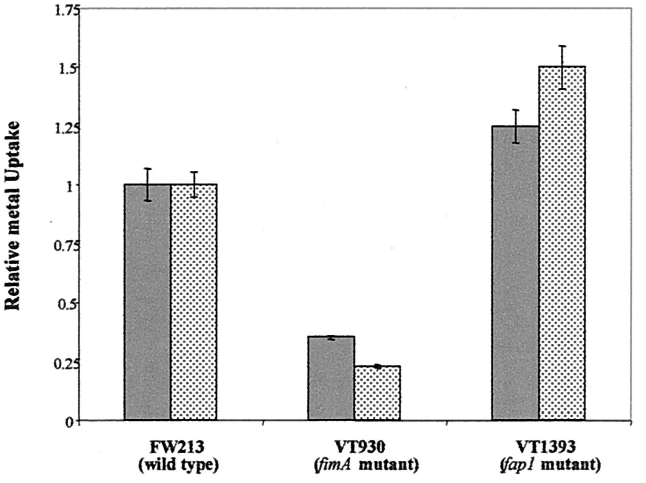

FIG. 6. Uptake of ^{54}Mn (grey bars) or ^{55}Fe (stippled bars) by wild-type FW213 and *fimA* and *fap1* mutant *S. parasanguis* strains. Cell-associated counts were corrected for cell number as determined by CFU analysis. All assays were done in duplicate; means and standard errors (error bars) are shown. This experiment was performed twice; a representative data set is shown.

growth differences between the wild-type and *fimA* mutant strains. These data suggest that FimA is a high-affinity transporter of Mn^{2+} and that there is at least one other low-affinity Mn^{2+} transporter in *S. parasanguis* FW213. These data also suggest that FimA is not required for the uptake of Fe^{3+} or Mg^{2+}, as growth of the *fimA* mutant is not affected by iron- or magnesium-limited conditions.

The *S. parasanguis fimA* mutant showed reduced metal uptake. Metal uptake in wild-type FW213 and the *fimA* mutant was compared (Fig. 6). Bacteria were grown overnight in TH broth containing either ^{55}Fe or ^{54}Mn, washed in fresh media containing an excess of either cold Fe^{3+} or Mn^{2+}, and radioactivity of the bacterial pellet was measured. Uptake of both isotopes was significantly lower in the *fimA* mutant; ^{55}Fe uptake was reduced by approximately 80%, while uptake of ^{54}Mn was reduced by approximately 65%. Growth of the *fimA* mutant was unaffected in TH broth, thus providing additional evidence that other metal transporters are present and functional. The *fap1* mutant strain VT1393, an isogenic mutant of FW213 lacking fimbriae, was not affected in its ability to take up ^{55}Fe or ^{54}Mn and serves as a control for this assay. The results of the uptake assay suggest that the ABC-type transporter encoded by the *S. parasanguis fimA* operon is a metal transporter with multiple specificities. This conclusion was supported by data from competition assays (Fig. 7) in which uptake of radiolabeled manganese or iron in wild-type FW213 was inhibited by the addition of excess unlabeled Fe^{3+} or Mn^{2+}.

Mn^{2+} concentration affects FimA expression, but not PepO expression. The expression of FimA in wild-type FW213 cells grown in the presence of different concentrations of divalent metals was examined by immunoblot analysis. Immunoblot analysis using FimA polyclonal antiserum indicated that FimA expression, as indicated by the presence of a 36-kDa band, was repressed in cells grown in media containing >0.1 μM Mn^{2+} (Fig. 8A, B, and C). Variation in the concentration of either Mg^{2+} or Fe^{3+} did not result in a change in FimA expression. PepO expression was unaffected by the range of Mn^{2+} concentrations that produced repression of FimA expression, as shown by the intensity of the 68-kDa band (Fig. 8D). These data were supported by the results of an enzyme-linked immunosorbent assay, which showed an approximate 25% reduction in FimA expression by cells grown in 10 μM Mn^{2+} compared to those cells grown in 0.1 μM Mn^{2+}, yet no detectable change in PepO expression occurred (data not shown).

DISCUSSION

Colonization of a host niche by bacteria requires delicate, highly regulated expression of proteins. Cues that trigger the expression of virulence factors tend to be simple environmental changes, such as nutrient availability, pH, and the presence of reactive oxygen species (17). The ability of the bacterium to sense its environment while in the host is paramount to its survival. It has been established that low concentrations of divalent metal ions can induce the production of various virulence traits, such as toxin production in *C. diphtheriae* in the case of low Fe^{2+} availability. The *C. diphtheriae* toxin regulator DtxR is a metalloregulator that is responsive to Fe^{2+} and Fe^{3+} and regulates the transcription of various genes involved in virulence by binding to a palindromic consensus sequence in the control regions of these genes (27). Palindromic operator

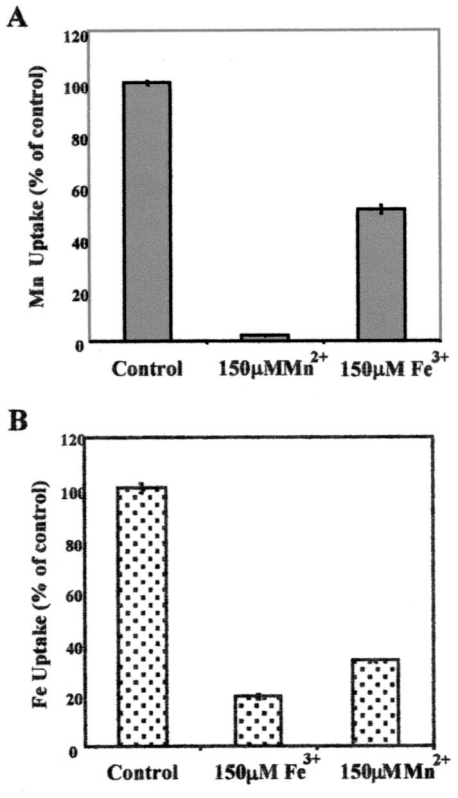

FIG. 7. Competition of manganese (A) and iron (B) uptake. The indicated amount of $MnCl_2$ or $FeCl_3$ was added to the uptake assay. All assays were done in duplicate; means and standard errors (error bars) are shown. This experiment was performed twice; a representative data set is shown.

elements which bind metallo-responsive regulators also exist in other bacterial species, such as the binding domains of *Staphylococcus epidermidis* SirR, *Mycobacterium tuberculosis* IdeR, *Treponema pallidum* TroR, *Bacillus subtilis* MntR, and *S. gordonii* ScaR (10, 18, 20, 30, 31). The *cis*-acting palindromic elements that bind the iron-responsive DtxR, SirR, and IdeR share homology. This homology, however, is not shared with the palindromic elements which bind the Mn^{2+}-responsive regulators TroR and MntR. ScaR is also a Mn^{2+}-responsive repressor, but its binding domain appears to belong to a family different from that of even TroR and MntR. The domain is characterized by a 46-bp region which includes two palindromic domains that are also present in the promoter regions of other members of the LraI family of permeases (20).

The *S. parasanguis fimA* operon encodes an ABC-type transport system belonging to the LraI family as indicated by DNA sequence homology (13). The *fimA* operon also possesses two palindromic domains that have 94 and 81% sequence identity to palindrome I and II, respectively, of the ScaR binding region of *S. gordonii* (20). The *S. parasanguis pepO* gene, which encodes a zinc metalloendopeptidase, is immediately upstream of and divergently transcribed from the *fimA* operon. Mapping of the *S. parasanguis fimA* operon by primer extension analysis revealed that the transcriptional start site of *fimA*, like that of the *S. gordonii sca* operon transcriptional start site, occurs within 16 nucleotides of the translational start site (1). Mapping of the *pepO* promoter showed that *pepO* has three transcriptional start sites, with the first starting within the *fimA* operon coding region. Mammalian ECE-1 also has multiple transcripts that differ in their 5′ untranslated regions as a result of alternative promoter usage (29). These data also indicated that the promoter and regulatory elements of the *fimA* operon and *pepO* gene overlap.

Analysis of the promoter activities of the *fimA* operon and *pepO* promoters under conditions of limiting Mn^{2+} availability indicated that the *fimA* operon is responsive to Mn^{2+} concentration. The responsiveness of the *fimA* operon promoter to Mn^{2+} availability suggests that the transcriptional regulation of the *fimA* operon is similar to the regulation of the *sca* operon of *S. gordonii* (20). In *S. gordonii*, regulation of transcription of the *sca* operon is determined by the binding of ScaR, the Mn^{2+}-responsive metallorepressor, to the *cis*-acting palindromic ScaR binding domain. In *S. parasanguis*, this putative repressor domain, also present in the promoter region of the *fimA* operon, did not affect the activity of the divergently transcribed *pepO* promoter as measured by luciferase activity. The *pepO* promoter, irrespective of Mn^{2+} concentration, showed luciferase activity levels 10,000 times greater than that associated with the *fimA* operon promoter. The high level of *pepO* promoter activity may be due to the cumulative effects of multiple *pepO* transcriptional start sites. In *S. parasanguis*, RNA stability, as well as promoter activity, may also play a role in the robust luciferase activity of the *pepO*-luciferase constructs. Computational analysis of the RNA folding of the *pepO* transcripts revealed the formation of a strong stem-loop structure in the 5′ untranslated region of the first and second transcripts, which could possibly protect the RNA from degradation by 5′ exonucleases (data not shown).

Further elucidation of the role of the *fimA* operon indicated that FimA is involved in the uptake of transition metal ions. Although FimA has specificity for at least two metal ions, Mn^{2+} and Fe^{3+}, evidence presented here suggests that FimA is involved primarily in high-affinity uptake of Mn^{2+}. Interestingly, the crystal structure of the PsaA metal binding site of the *S. pneumoniae* ABC-type transporter reveals that the active site was associated with Zn^{2+}, although in vivo studies suggest that it is predominantly a Mn^{2+} transporter (9, 26). The ability of FimA to transport both Mn^{2+} and Fe^{3+} may be explained by the active site having a greater affinity for Mn^{2+}, but conformational shifting of the solute binding domain can accommodate the binding of other divalent metal ions.

In summary, the *fimA* operon of *S. parasanguis* encodes a metal transporter with activity similar to those of other members of the LraI family. Transcription and expression of these genes are responsive to Mn^{2+} availability, but not to Fe^{3+} or Mg^{2+} availability. The divergently transcribed *pepO* does not show the same response to metal availability as the *fimA* operon, which suggests that the two are not coordinately regulated under these conditions.

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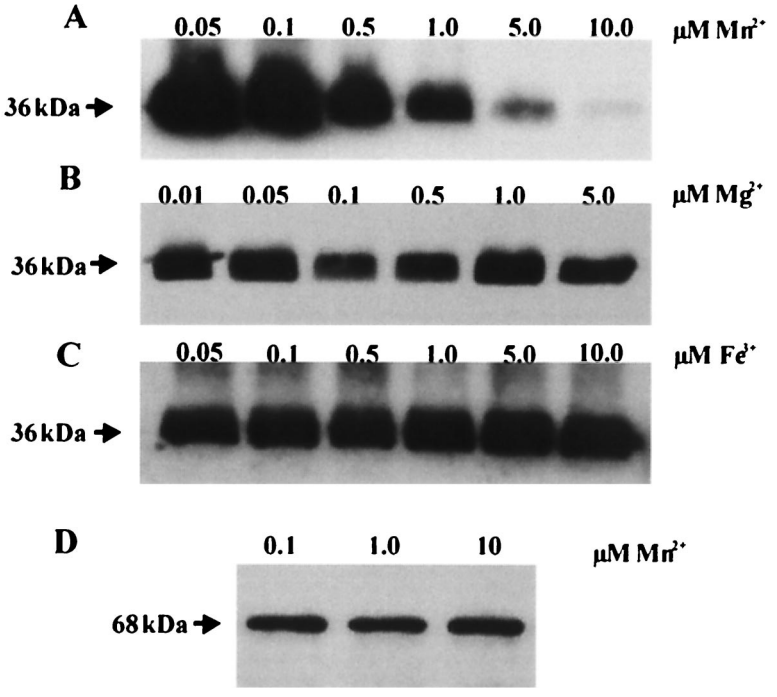

FIG. 8. Immunoblot detection of FimA expression in wild-type FW213 cells grown in Chelex-100-treated CDM supplemented with metal ions. The cells were grown with the following concentrations of Mn^{2+}, Mg^{2+}, and Fe^{3+}: Mn^{2+} (0.05 to 10 μM), 5.0 μM Mg^{2+}, and 1.0 μM Fe^{3+} (A); Mg^{2+} (0.01 to 5.0 μM), 1.0 μM Fe^{3+}, and 1.0 μM Mn^{2+} (B); and Fe^{3+} (0.05 to 10 μM), 5.0 μM Mg^{2+}, and 1.0 μM Mn^{2+} (C). Proteins were extracted from an equal number of cells as measured by OD_{490}, subjected to SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and probed with polyclonal antiserum directed toward FimA. (D) Immunoblot detection of PepO expression in wild-type FW213 cells grown in Chelex-100-treated CDM supplemented with Mn^{2+} (0.1, 1.0, or 10 μM), 5.0 μM Mg^{2+}, and 0.1 μM Fe^{3}. One microgram of total protein per lane was subjected to SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and probed with polyclonal antiserum directed toward PepO.

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