JOURNAL OF BACTERIOLOGY, Oct. 2003, p. 5967–5975 Vol. 185, No. 20
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.20.5967–5975.2003

The *sloABCR* Operon of *Streptococcus mutans* Encodes an Mn and Fe Transport System Required for Endocarditis Virulence and Its Mn-Dependent Repressor

Sehmi Paik,[1,2] Arunsri Brown,[1] Cindy L. Munro,[3] Cynthia Nau Cornelissen,[2] and Todd Kitten[1,2]*

The Philips Institute of Oral and Craniofacial Molecular Biology,[1] Department of Microbiology and Immunology,[2] and Department of Adult Health Nursing,[3] Virginia Commonwealth University, Richmond, Virginia 23298

Received 14 July 2003/Accepted 29 July 2003

Streptococcus mutans **belongs to the viridans group of oral streptococci, which is the leading cause of endocarditis in humans. The LraI family of lipoproteins in viridans group streptococci and other bacteria have been shown to function as virulence factors, adhesins, or ABC-type metal transporters. We previously reported the identification of the *S. mutans* LraI operon, *sloABCR*, which encodes components of a putative metal uptake system composed of SloA, an ATP-binding protein, SloB, an integral membrane protein, and SloC, a solute-binding lipoprotein, as well as a metal-dependent regulator, SloR. We report here the functional analysis of this operon. By Western blotting, addition of Mn to the growth medium repressed SloC expression in a wild-type strain but not in a *sloR* mutant. Other metals tested had little effect. Cells were also tested for aerobic growth in media stripped of metals then reconstituted with Mg and either Mn or Fe. Fe at 10 μM supported growth of the wild-type strain but not of a *sloA* or *sloC* mutant. Mn at 0.1 μM supported growth of the wild-type strain and *sloR* mutant but not of *sloA* or *sloC* mutants. The combined results suggest that the SloABC proteins transport both metals, although the SloR protein represses this system only in response to Mn. These conclusions are supported by ^{55}Fe uptake studies with Mn as a competitor. Finally, a *sloA* mutant demonstrated loss of virulence in a rat model of endocarditis, suggesting that metal transport is required for endocarditis pathogenesis.**

Streptococcus mutans is a gram-positive aerotolerant anaerobe residing in the oral cavity. *S. mutans* uses host dietary sucrose as well as other carbon sources for cellular energy requirements. It does not possess cytochromes and generates energy via glycolysis, liberating mixed acids as fermentation products (23). This acid production contributes to smooth-surface dental caries pathogenesis by *S. mutans* (23). The viridans group streptococci, including *S. mutans*, also cause human endocarditis, accounting for 45 to 80% of all cases involving native valves (5, 65).

Infective endocarditis is a life-threatening endovascular infection believed to occur when bacteria in the bloodstream adhere to previously damaged heart valves (16). Endocarditis causes substantial morbidity and mortality despite improvements in medical and surgical treatment (16). Prevention efforts are confined to antibiotic prophylaxis for invasive dental or surgical procedures that are likely to produce bacteremia (13). Although currently recommended by the American Heart Association (13), the merit of antibiotic prophylaxis for dental procedures has been much debated (1, 54, 57, 60). A vaccine would be a preferable prophylactic for many reasons (4). Recent vaccine efforts have focused on the FimA protein, an LraI (lipoprotein receptor antigen I) protein from *Streptococcus parasanguis* (35, 66).

LraI family proteins were initially recognized in oral streptococci and have been identified in other bacteria (31, 34, 61). This family has since been extended (11). These proteins have several common features. The LraI genes are the constituents of operons encoding ATP-binding cassette (ABC) transport systems. In gram-positive bacteria, the basic components of these operons are genes for an ATP-binding protein (ATPB), an integral membrane protein (IMP), and an LraI protein (11, 18, 24). The LraI proteins have homology to the periplasmic substrate-binding proteins of gram-negative bacterial ABC transport systems and have been proposed to possess transporter functions (2, 15, 21). In addition, adhesin functions of LraI proteins have been demonstrated in *Streptococcus sanguis* (SsaB) (20), *Streptococcus gordonii* (ScaA) (37), *S. parasanguis* (FimA) (50), and *Streptococcus agalactiae* (Lmb) (59). However, in a recent reevaluation, inactivation of the *sca* genes has been observed to have no effect on coaggregation with *Actinomyces naeslundii* (28). Lmb has been shown to be required for adherence to human laminin, but a typical ABC transporter has not been found in the nucleotide sequence adjacent to the *lmb* locus (19, 59), and the addition of manganese did not affect the growth of an Lmb mutant (19, 59). These studies suggest that LraI proteins may possess adhesin or metal transporter functions or both.

In our previous study (34), an LraI family operon, *sloABCR*, was characterized in *S. mutans*, and it was proposed that the *sloABC* genes encode the ATP-binding protein, integral membrane protein, and solute-binding lipoprotein, respectively, of a high-affinity Mn transport system. This hypothesis was based on the homology of these genes to LraI operons, such as the *scaBCA* system in *S. gordonii*, which has been shown to encode an Mn transport system (36). We also proposed that SloR

* Corresponding author. Mailing address: The Philips Institute of Oral and Craniofacial Molecular Biology, 521 North 11th St., Box 980566, Virginia Commonwealth University, Richmond, VA 23298-0566. Phone: (804) 628-7010. Fax: (804) 828-0150. E-mail: tkitten@vcu.edu.

TABLE 1. Bacterial strains and plasmids used in this study

S. mutans strain or plasmid	Genotype or phenotype	Source or reference
Strains		
V403	Human blood isolate, fructan-hyperproducing, cariogenic strain	33, 46, 48, 56
V2613	Δ*sloC1*, derived from V403	34
V2643	Kan^r, Δ*sloR1*::*aphA-3*, derived from V403	This study
JFP14	Kan^r, *sloA1*::*aphA-3*, derived from V403	This study
Plasmids		
pVA2570	Ap^r; 7.63 kb, *sloC*, *sloR*, and downstream flanking DNA	34
pVA2587	Ap^r; 8.14 kb, *sloABCR* operon and flanking DNA	34
pVA2592	Ap^r Kan^r; This study containing 1.37-kb *aphA-3* cassette ligated at *Bam*HI site	pUC19
pVA891	Em^r Cm^r; 6.45 kb, suicide vector	41
pVA838	Em^r Cm^r; 9 kb, *E. coli–Streptococcus* shuttle plasmid	42
pVA2642	Kan^r Em^r; 5.9-kb *Eco*RV-*Sal*I fragment containing Δ*sloR1::aphA-3* ligated into pVA891	This study
pJFP9	Cm^r Em^r; 1.03-kb *Xmn*I fragment containing *sloR* from pVA2587; ligated into pVA838	This study
pVA2587-kan	Ap^r Kan^r; 1.37-kb *aphA-3* cassette inserted at *Eco*NI site in the *sloA* gene in pVA2587	This study

functions as a metal-dependent regulator based on its homology to MntR homologs in other gram-positive bacteria (25, 28, 32, 51–53). It was therefore surprising when Spatafora et al. (58) working with another *S. mutans* strain reported that the *sloABC* genes (renamed *ORF1*, *ORF2*, and *fimA*) encode an Fe transport system and that SloR (renamed Dlg) functions as an Fe-dependent repressor for these genes.

In this study, we report the functional characterization of the s*loABC* genes in *S. mutans* V403 and their manganese-dependent regulation by SloR. We also further define the role of the operon in endocarditis virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. V403 is a fructan-hyperproducing, cariogenic strain of *S. mutans* (33, 45, 48, 56) originally isolated from human blood. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for cloning, and cultures were routinely grown at 37°C shaking in Terrific broth (TB) (62). Erythromycin was added at 300 μg/ml, and kanamycin was added at 50 μg/ml as needed for plasmid selection. *S. mutans* was grown in anaerobic conditions at 37°C without shaking in brain heart infusion (BHI) broth (Difco, Inc., Detroit, Mich.) supplemented with 1.5% agar for growth on plates, unless otherwise indicated. Erythromycin was included at 10 μg/ml and kanamycin was included at 100 μg/ml for streptococcal transformant selection. Genetic transformation was performed as previously described by Lindler and Macrina (39).

DNA manipulations. Chromosomal DNA was isolated from *S. mutans* as described previously (34). Southern blot labeling and detection were performed with the Genius digoxigenin system (Roche Molecular Biochemicals, Indianapolis, Ind.). PCR was routinely performed in a GeneAmp 960 thermal cycler (PE Biosystems, Foster City, Calif.) with Platinum PCR Supermix (BRL). Oligonucleotide primers were synthesized by BRL. Restriction enzymes were purchased from BRL or New England Biolabs, Inc., Beverly, Mass.) and used according to the manufacturer's instructions.

Construction of mutant strains. (i) *sloR* mutant. The *sloR* mutant was constructed as follows. Two rounds of PCR were performed to generate a deletion marked by a *Bam*HI site in *sloR* as described previously (26, 34). *Sal*I and *Eco*RV sites were incorporated at either end of each amplicon for cloning. P1 (5′-CCA TAGATATCGTTCCTGTTGGTC-3′) and P2 (5′-AGCCGGATCCATCTCGT TCACTGAG-3′) were used for one amplicon. P3 (5′-GTGATGGATCCGGC TGTCTCAGAGAT-3′) and P4 (5′-GCGTCGACCTTATTACCTTCGC-3′) were used for the other. The underlined sequences indicated the restriction sites *Eco*RV (P1), *Bam*HI (P2 and P3), and *Sal*I (P4). PCR products were purified from an agarose gel with a Freeze'n Squeeze DNA gel purification kit (Bio-Rad, Hercules, Calif.). The second round of PCR using P1 and P4 generated a sequence with an internal *Bam*HI site as well as a 46-bp deletion in *sloR*. This 1.89-kb fragment was ligated into the *Eco*RV and *Sal*I sites of pVA891. The resulting plasmid was digested with *Bam*HI. The *aphA-3* cassette from pVA2592 digested with *Bam*HI was ligated into the *Bam*HI-digested plasmid to disrupt *sloR* and to insert a selectable marker. The final suicide vector was designated pVA2642. This was linearized with *Sca*I and used to transform *S. mutans* V403. Selection with kanamycin resulted in isolation of a *sloR* mutant created by allelic exchange, V2643.

(ii) *sloA* mutant. The plasmid pVA2587 (34) was digested with *Eco*NI, and the sticky ends resulting from the digestion were filled in by using deoxynucleoside triphosphates (dNTPs) and the large fragment of DNA polymerase I (BRL) to create blunt ends. The *aphA-3* cassette was obtained from pVA2592 by *Bam*HI digestion, made blunt as described above, and ligated into pVA2587 to disrupt the *sloA* gene. The final suicide vector was designated pVA2587-kan. It was linearized with *Sca*I for transformation of *S. mutans* V403 to create strain JFP14.

(iii) *sloR*-complemented strains. A plasmid expressing the *sloR* gene was constructed. Plasmid pVA2570 (34) was digested with *Xmn*I, which produced four DNA bands. The 1,030-bp fragment that contained the *sloR* gene was gel purified and cloned into pVA891 at the *Nru*I site. The resulting plasmid was designated pJFP9 and was used to transform V2613 (34) and V2643.

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% Criterion precast gels (Bio-Rad), unless otherwise indicated, and visualized with Coomassie brilliant blue. Western blotting of duplicate unstained gels and immunodetection of SloC with anti-FimA antiserum were performed as described previously (66). Western blots were visualized with anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase (AP) conjugated antibody (Promega, Madison, Wis.) and Western blue stabilized substrate for AP (Promega), unless otherwise indicated.

Growth studies. BHI broth was treated to limit the availability of metal ions. Five grams of Chelex 100 (Bio-Rad) per 200 ml of BHI broth was prewashed in 100 ml of deionized water three times and then added to dissolved BHI, followed by stirring for 90 min at room temperature. Resin beads were removed, and the medium was filter sterilized. Magnesium sulfate was added to the Chelex-treated BHI broth at 81 or 810 μM. This basal medium was designated BCMg81 or BCMg810, respectively. Overnight cultures grown anaerobically in BHI were diluted 1,000-fold into fresh BHI broth, BCMg81, or BCMg81 supplemented with $MnCl_2$, $Fe(III)C_6H_5O_7$, or $Fe(II)SO_4$. Duplicate cultures were aerobically incubated at 37°C for up to 48 h without shaking.

^{55}Fe uptake study. For ^{55}Fe uptake experiments, FMC media (63) were treated with Chelex 100 as described above and reconstituted with 810 μM Mg (FMC-CMg810). Overnight cultures of *S. mutans* strains were anaerobically grown in anaerobically preincubated FMC-CMg810. Cultures were then diluted 25- to 75-fold into anaerobically preincubated FMC-CMg810 and incubated for an additional 5 to 6 h. One culture of each strain with an optical density at 660 nm (OD_{660}) of approximately 0.7 to 0.8 was selected for the uptake study. $^{55}FeCl_3$ (2 mCi; specific activity, 18.1 mCi/mg) was purchased from Perkin-Elmer/NEN (Boston, Mass.), and 0.4 μCi was used for each sample. The MultiScreen assay system (Millipore, Bedford, Mass.) was used for the uptake experiments. The filtration 96-well plate was prewetted with 100 μl of 0.1 M $NiSO_4$ to prevent nonspecific binding of Fe to the filter membranes. A metal mix solution, including 0.4 μCi of $^{55}FeCl_3$, $FeSO_4$, and, for some experiments, $MnCl_2$, was prepared. Twenty microliters of the metal mix solution was mixed

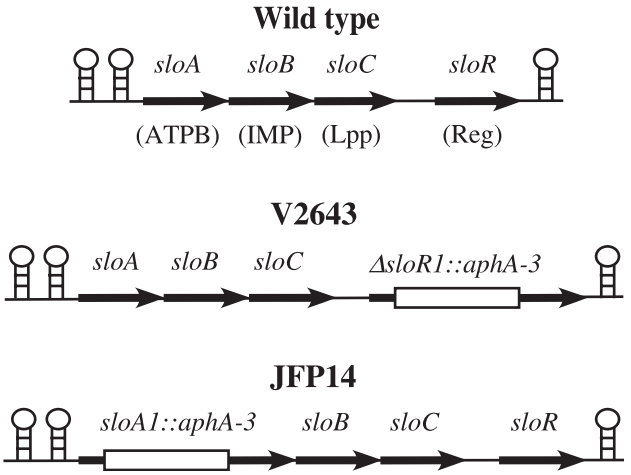

FIG. 1. *sloR* and *sloA* mutant strains. The *sloABCR* operon is shown at the top with the genes depicted by arrows. DNA sequences containing inverted repeats are shown as stem-loops. (See GenBank accession no. AF232688.) Putative functions for the genes are indicated: ATPB, ATP-binding protein; IMP, integral membrane protein; Lpp, lipoprotein receptor; Reg, metal-dependent regulator. V2643 contains a 46-bp deletion and a 1.4-kb *aphA-3* cassette encoding resistance to kanamycin inserted into the *sloR* gene in the same orientation as *sloR*, as shown by the open box. JFP14 contains the *aphA-3* cassette inserted into the *sloA* gene in the same orientation.

with 480 μl of cells in microcentrifuge tubes. The final concentrations of each metal in the sample were as follows: ^{55}Fe at 0.27 μM; Fe at 0, 1, 5, 10, and 15 μM; and Mn at 1, 4, 8, and 12 μM. In addition, sodium ascorbate was added to a final concentration of 5 mM (17). Samples were incubated for periods ranging from 2 to 30 min at room temperature. Immediately thereafter, 200 μl of cells was transferred to the 96-well plate, and the liquid was filtered out by vacuum. Free ^{55}Fe was removed by three successive washes with 200 μl of citrate buffer (100 mM $Na_3C_6H_5O_7$, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$). Parallel experiments were performed with cells incubated on ice as a nonspecific binding control. The cpm values obtained for chilled cells (which remained constant over time) were subtracted from those obtained from room temperature-incubated cells. Filters were air dried in a fume hood. A MultiScreen punch (Millipore) was used to isolate each filter from the 96-well plate, which was collected in a high-density polyethylene scintillation vial (Fisherbrand; Fisher, Pittsburgh, Pa.). Four milliliters of scintillation fluid was added to the vial, and the radioactivity was measured by scintillation counting with "wide open" window setting, using a Beckman LS6500 scintillation counter (Fullerton, Calif.).

Animal models. The previously described rat model of endocarditis was employed (47). The protocol received Institutional Animal Care and Use Committees approval (no. 0010-2865) and complied with all applicable federal guidelines and institutional policies. In brief, a catheter was inserted through the internal carotid artery past the aortic valve to impose valve damage. The catheter was sutured and remained in the artery throughout the rest of the experiment. Two days later, streptococci grown in BHI broth plus 0.5% sucrose were harvested, washed in phosphate-buffered saline (PBS), and inoculated into the tail vein of the catheterized rats. Two days later, the rats were sacrificed by CO_2 inhalation. The heart was removed, and accurate catheter placement was assessed visually. The aortic valve and any apparent vegetations were removed, homogenized with PBS, and spread on tryptic soy agar plates. Rats were judged to be infected if bacteria were recovered. Rats in which correct catheter placement could not be verified at necropsy, which had no apparent vegetations, and from which no bacteria were recovered were dropped from the study. All other rats from which bacteria were not recovered were judged to be uninfected. Differences in infectivity were evaluated by using the exact Cochran-Mantel-Haenzel test and Fisher's exact test.

RESULTS

SloR is an Mn-dependent repressor of SloC expression. In our previous study (34), the DNA sequence of an LraI operon, *sloABCR*, from *S. mutans* was determined. The structure of this operon is shown in Fig. 1. We hypothesized that the *sloR* gene product functions as a metal-dependent repressor of *sloABCR* expression. To test this hypothesis, a *sloR* mutant was constructed for this study (V2643; Fig. 1).

Western blot analysis was performed with the wild-type strain (V403), a *sloR* mutant (V2643), and a Δ*sloC* mutant (V2613). The effects of Mn and Fe on SloC expression were investigated. Figure 2 shows Western blots examining cultures grown with variable concentrations of Mn or Fe added to the culture medium (BHI). A band with a molecular mass of 34 kDa reacted with a rabbit polyclonal anti-FimA antiserum for both the wild type (V403) and the *sloR* mutant (V2643) in each Western blot. This band was not detected in the Δ*sloC* mutant (V2613), indicating that this protein is SloC. In the presence of 50 and 500 μM $MnSO_4$, SloC expression in V403 (wild type) was repressed (Fig. 2A). In contrast, no significant repression was observed with V403 at 50 or 500 μM $FeSO_4$, and only partial repression was observed at 1,000 μM $FeSO_4$ (Fig. 2A). SloC expression in the *sloR* mutant (V2643) remained high regardless of the amount of added Mn^{2+} or Fe^{2+} (Fig. 2A). This suggests that Mn^{2+}-dependent repression of SloC is mediated by the SloR protein.

It seemed possible that the form of Fe used for the experiment could be important. Therefore, both the Fe^{2+} and Fe^{3+} forms of iron were separately tested by Western blot analysis, as shown in Fig. 2B. In addition, lower concentrations of Fe and Mn were used. Slight repression of SloC was observed at

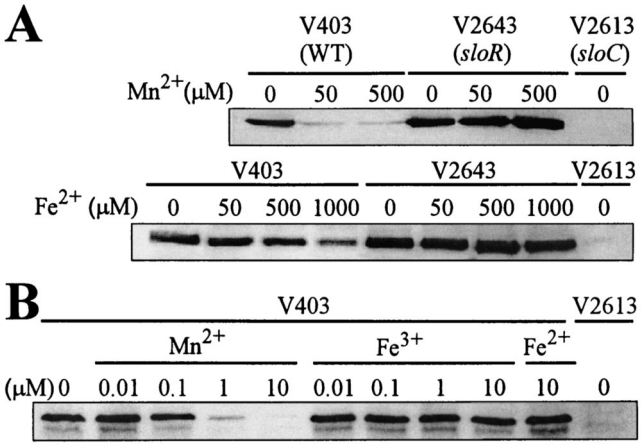

FIG. 2. Western blot analysis of the effect of Mn and Fe on SloC expression. Equal amounts of total protein isolated from cultures of *S. mutans* V403 (wild-type), V2643 (*sloR* mutant), and V2613 (Δ*sloC* mutant) were separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane for Western blot detection with rabbit polyclonal anti-FimA antiserum. (A) Overnight cultures grown in BHI broth under anaerobic conditions were diluted 100-fold into fresh BHI broth or BHI broth supplemented with $MnSO_4$ or $FeSO_4$ added at the concentrations indicated. Protein preparations were made the following day after overnight growth as described previously (66). Gels were 12% Bio-Rad Ready cast, and the ECL enhanced chemiluminescence system (Amersham, Piscataway, N.J.) was used for visualization of Western blots. (B) Cultures were grown anaerobically in BHI broth with $MnCl_2$, $Fe(III)C_6H_5O_7$, or $Fe(II)SO_4$ at the concentrations indicated. Lysates were prepared as described above, except that a Fast Prep, FP120 (Bio101, Savant, Vista, Calif.) bead beater was used at level 6 for 30 s to lyse cells.

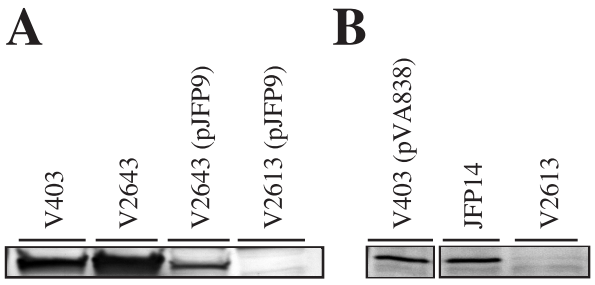

FIG. 3. Western blot analysis of SloC expression in mutant strains. Streptococcal lysates from the indicated strains grown anaerobically in BHI were analyzed by Western blotting with anti-FimA antiserum. V403, wild type; V2613, Δ*sloC* mutant. (A) V2643, *sloR* mutant; V2643 (pJFP9), *sloR*-complemented *sloR* mutant; V2613(pJFP9), *sloR*-complemented *sloC* mutant. (B) Separate blot examining JFP14, *sloA* mutant.

0.1 μM Mn^{2+}, and SloC was strongly repressed by 10 μM Mn^{2+}. In contrast, neither form of iron changed the level of SloC detected by Western blotting (Fig. 2B). When six additional metals ($ZnCl_2$, $CuSO_4$, $NiSO_4$, $MgCl_2$, $CoCl_2$, and $CaCl_2$) were tested at 50 and 500 μM concentrations, none appeared to affect SloC expression in V403, although 500 μM $CuSO_4$ appeared to slightly inhibit growth (data not shown).

Role of the *sloABCR* operon in growth under metal-limited conditions. Based on the results shown in Fig. 2 and the homology of the *sloABC* gene products to other Mn transport systems, it seemed reasonable that the SloABC system would transport Mn. However, because Spatafora et al. (58) reported this system functions in the transport of Fe and not Mn, both metals were investigated for uptake. It was shown previously that Mg is the only metal required by *S. mutans* for growth under anaerobic conditions (44). Under aerobic conditions, either Fe or Mn was required in addition to Mg. Our growth studies with V403 and its derivatives indicated that they also required only Mg when grown under anaerobic conditions (data not shown). These results led us to reason that if Mn or Fe were essential for *S. mutans*, this requirement would be revealed under aerobic growth conditions. As one test of whether the *sloABCR* operon encoded an ABC-type Mn or Fe uptake system, wild-type and mutant strains were grown aerobically in metal-limited media.

In preparation for growth studies, additional mutant strains were constructed. To confirm that the derepression of SloC in V2643 resulted from loss of *sloR*, a *sloR*-containing plasmid, pJFP9, was introduced into V2643. The pJFP9 plasmid was also introduced into V2613. In our previous study (34), it was not clear whether *sloR* was expressed in V2613. The expression of the *sloR* gene from a plasmid could separate possible effects of *sloC* mutation from a *sloR* mutation. The production of SloR by pJFP9 in the two complemented strains was assessed indirectly by Western blot analysis (Fig. 3A). The level of SloC expression in V2643(pJFP9) was reduced compared to that in V2643. This result would be expected if SloR is being produced from pJFP9 and repressing *sloC* transcription. Since SloC expression is even lower in V2643(pJFP9) than in V403 (Fig. 3A), it appears that SloR is produced at greater than wild-type levels from pJFP9. Although SloR expression from pJFP9 could not be assessed in V2613(pJFP9), because of the lack of SloC production in that strain (Fig. 3A), it is reasonable to assume that SloR was also expressed in that strain.

A *sloA* mutant was constructed by inserting the *aphA*-3 cassette into the *sloA* gene, and the new strain was designated JFP14 (Fig. 1). To determine whether this mutation was polar on expression of downstream genes in the operon, SloC expression in JFP14 was investigated by Western blot analysis. The results are shown in Fig. 3B. SloC expression in this strain is equivalent to that in the wild-type strain, V403. Transcription of *sloC* presumably originates either from the *aphA*-3 promoter or from read-through transcription, which has been observed previously with this cassette (18).

V403, V2613, V2613(pJFP9), V2643, V2643(pJFP9), and JFP14 were grown aerobically in BHI or BCMg81 medium supplemented with either Fe (Fe^{3+} or Fe^{2+}) or Mn^{2+}. Growth was not observed in any strain in BCMg81 (data not shown), whereas all strains grew equivalently in BHI (Fig. 4A). At 10 μM, Fe^{3+} partially restored growth of V403 and V2643 (pJFP9), although it was delayed compared to growth in BHI broth (Fig. 4B). In some experiments, V2643 exhibited poor growth: up to 21% of its growth in BHI broth (data not shown). Growth of the *sloA* or Δ*sloC* strains was not detected throughout the experiment. The failure of the *sloA* and Δ*sloC* mutants to grow suggests that the SloABC proteins function in Fe uptake. The reason for the failure of the *sloR* mutant, V2643, to grow well was not immediately apparent. Interestingly, V2643(pJFP9) grew more quickly than V403 (Fig. 4B). One explanation for both of these findings was that Fe was required for growth in the absence of other metals, but too much Fe was toxic. The level of *sloABC* expression in V2643 and V403 may have led cells to take up too much iron, while overproduction of SloR in V2643(pJFP9) may have led to near-optimal Fe uptake. To test this possibility, V403, V2613, JFP14, and V2643 were grown at lower Fe^{3+} concentrations (Fig. 4C). V403 again grew in 10 μM Fe^{3+}; however, growth was not improved by lowering the Fe concentration. With 10 μM Fe^{2+} ($FeSO_4$), V403, V2643, and V2643(pJFP9) exhibited poor and inconsistent growth (0 to 20% compared to their growth in BHI), whereas the *sloA* and Δ*sloC* mutants showed no growth (data not shown).

When Mn^{2+} was added at 10 μM, growth of V403 and all the mutant strains was equivalent to that shown in Fig. 4A for BHI broth (data not shown). This was not unexpected, inasmuch as maximal growth of the *scaA* and *scaC* LraI operon mutants of *S. gordonii* was restored by the addition of ≥1 μM Mn^{2+} (36). The effect of addition of lower concentrations of Mn^{2+} was therefore investigated (Fig. 4D). At Mn^{2+} concentrations of 0.01 and 0.1 μM, V2613 and JFP14 showed no detectable growth ($OD_{600} \leq 0.01$ at 16 h), whereas at Mn^{2+} concentrations of 0.01 and 0.1 μM, V403 and V2643 attained ODs of 0.07 to 0.20 and 0.49 to 0.56, respectively. At 1 μM Mn^{2+}, V2613 and JFP14 grew to intermediate densities, and at 10 μM, the growth of JFP14 was restored to that of the wild type, while V2613 approached this level (Fig. 4D). When the OD_{600} was measured at later time points (18 and 20 h), 0.01 and 0.1 μM concentrations of Mn^{2+} still did not support the growth of V2613 and JFP14 (data not shown). These results suggest that the SloABC system functions as a high-affinity Mn^{2+} transport system that is essential for aerobic growth of

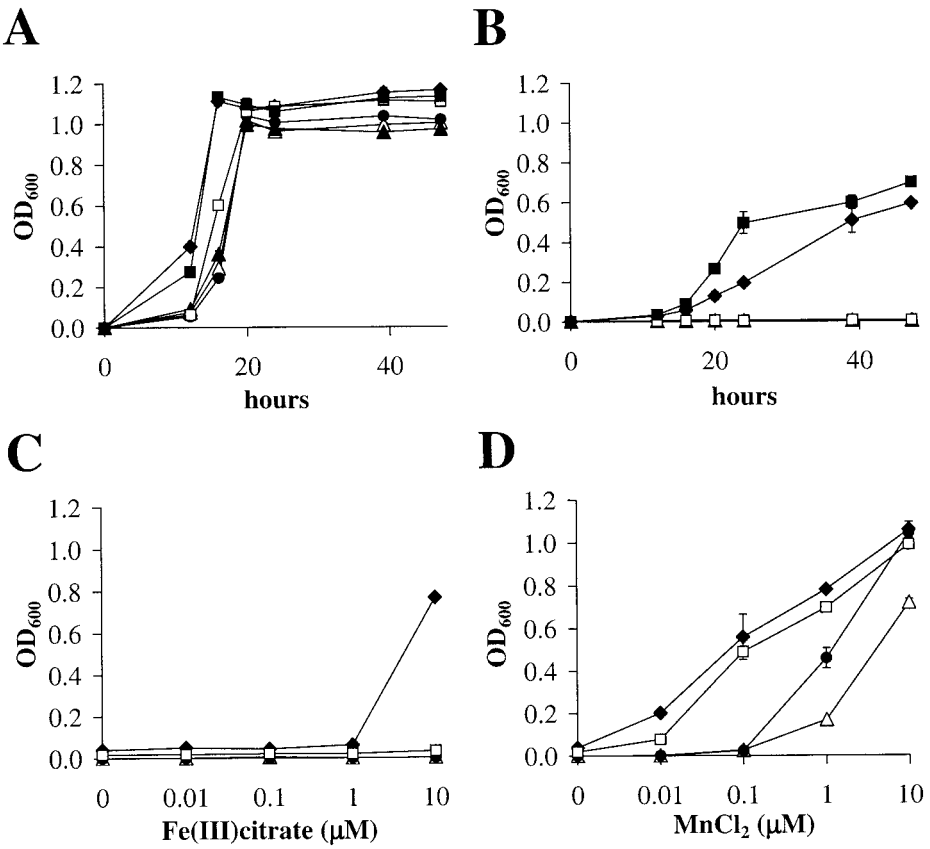

FIG. 4. Aerobic growth of wild-type and mutant strains of *S. mutans*. (A and B) Growth in (A) BHI broth and BCMg81 plus 10 μM Fe(III) $C_6H_5O_7$ (B). OD_{600} was measured at 0, 12, 16, 20, 24, 39, and 47 h of incubation. (C and D) Culture densities at a single time point in BCMg81 containing Fe(III) $C_6H_5O_7$ or $MnCl_2$ at the concentrations indicated. (C) OD_{600} at 38 h of incubation with Fe(III) $C_6H_5O_7$. (D) OD_{600} at 16 h of incubation with $MnCl_2$. ◆, wild-type; △, *sloC* mutant; ▲, *sloR*-complemented *sloC* mutant; ●, *sloA* mutant; □, *sloR* mutant; ■, *sloR*-complemented *sloR* mutant. The two *sloR*-complemented strains were not included in panels C and D. Error bars indicate standard deviations from duplicate cultures.

S. mutans under low (0.01 and 0.1 μM) Mn^{2+} concentrations and that there is at least one additional, lower-affinity Mn^{2+} transport system in *S. mutans.* The combined results also suggest that Mn supports aerobic growth more efficiently than Fe in *S. mutans*.

The SloABC system functions in Fe and Mn acquisition. A more direct approach was employed to investigate Fe and Mn acquisition by the SloABC system. Wild-type and mutant strains were examined for acquisition of radioactive iron with or without nonradioactive Mn as a competitor. First, ^{55}Fe acquisition by V403 (wild type) over 2 to 30 min was measured. The uptake velocity was greatest at 2 min and decreased at longer intervals, as expected (data not shown). Next, uptake velocities for V2613 (Δ*sloC*), JFP14 (*sloA*), and V2643 (*sloR*) were compared to that of V403 over a period of 2 min (Fig. 5A). The graphs for V403 (wild type) and V2643 (*sloR*) show saturation kinetics, as expected for active transport, whereas those for V2613 (Δ*sloC*) and JFP14 (*sloA*) are barely above background.

As shown in Table 2, the V_{max} of V2643 (*sloR*) is about five times that of V403 (wild type), whereas the K_m values of the two strains are similar. This is the expected result if Fe uptake occurs by the same transporter in both strains, with the transporter more abundant in the *sloR* mutant. The amount of Fe acquired by V2613 (Δ*sloC*) or JFP14 (*sloA*) was too low to produce reliable K_m or V_{max} values, as evidenced by the large standard errors for these strains. The average velocities for V2613 (Δ*sloC*) and JFP14 (*sloA*) from three experiments were 9.3 and 6.8%, respectively, of that of the wild type at 5.27 μM Fe. These findings support the conclusion that the SloABC system is involved in the acquisition of Fe in *S. mutans* and that, in the absence of SloR repression, increased expression of SloABC results in increased Fe acquisition.

Next, Mn was added as a competitive inhibitor of ^{55}Fe uptake. If Mn is transported by the same system that transports Fe, the apparent K_m for Fe, $K_m^{App\ (Fe)}$, should increase with increasing Mn. By plotting $K_m^{App\ (Fe)}$ against [Mn], the concentration of Mn that causes half-maximal inhibition of Fe uptake, K_i^{Mn}, can be determined. K_m^{app} values for Fe uptake were measured in the presence of Mn concentrations of 1, 4, 8, and 12 μM, as shown in Fig. 5B. The slope of the best-fit line is 3.6 ± 0.8, which represents K_m/K_i. The *x*-intercept is −2.0, and the *y*-intercept is 7.0 ± 6.0, which represent $-K_i^{Mn}$ and K_m^{Fe}, respectively. The *y*-intercept, although containing a large standard error, is close to the K_m^{Fe} value of V403 shown in Table 2. The finding that K_i^{Mn} (2.0 μM) is lower than K_m^{Fe} and that the slope (K_m^{Fe}/K_i^{Mn}) is larger than 1 suggests that the SloABC system has a higher affinity for Mn than Fe.

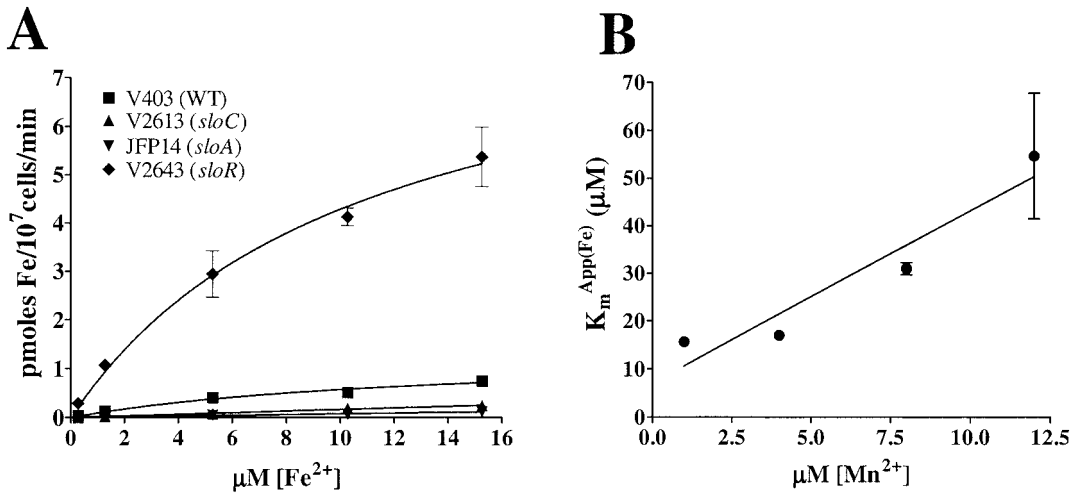

FIG. 5. ^{55}Fe acquisition by *S. mutans*. (A) Acquisition of radioactive Fe over 2 min in wild-type and *slo* mutant strains of *S. mutans* at five different Fe concentrations (0.27, 1.27, 5.27, 10.27, and 15.27 μM). Points are connected by nonlinear regression lines. Experiments were performed three times. The results shown are from one representative experiment. (B) Apparent K_m values for Fe acquisition in the presence of Mn as a competitor at 1, 4, 8, and 12 μM in wild-type *S. mutans* (V403). Samples were prepared in quadruplicate except those with 12 μM Mn (duplicate). The experiment was performed twice with similar results. One data set connected by a linear regression line is shown.

***A sloA* mutant has reduced virulence in a rat endocarditis model.** V403 and the *sloA* mutant, JFP14, were tested for virulence in a rat model of endocarditis. The results are shown in Table 3. In experiment 1, V403 caused infection in 5 of 11 rats, whereas JFP14 infected 0 of 7 rats. The 45% infection rate obtained for V403 is similar to that seen for this strain in previous experiments (34); nevertheless, it was hoped that this infection rate could be increased by employing a more rigid catheter material. For experiment 2, which was performed at the same time as experiment 1, a monofilament catheter material was used. In this experiment, V403 caused 50% infection, and again, JFP14 infected no rats. The catheter type therefore showed no apparent effect on infection rate by either strain. The results of the two experiments were combined and tested by the Cochran-Mantel-Haenzel test. The infection rate of 7 of 15 (47%) for V403 was significantly greater than the infection rate of 0 of 11 (0%) for JFP14 ($P = 0.0231$; Table 3, combined).

DISCUSSION

The contribution of SloR to SloC expression in *S. mutans* was examined. Figure 2 shows that SloC is repressed when cells possessing a functional *sloR* gene are grown in the presence of Mn. This suggests that SloR is an Mn-dependent repressor of SloC expression. No other metal tested repressed SloC expression efficiently. When Fe was added at 1 mM, SloC was slightly repressed (Fig. 2A). However, this could be due to contamination by Mn in the $FeSO_4$ added to the media. According to the manufacturer's analysis, Mn was present at 0.03%, indicating a concentration of approximately 0.5 μM in a 1 mM solution of $FeSO_4$. Examination of Fig. 2B suggests that this concentration of contaminating Mn could account for the degree of repression observed in Fig. 2A. It is not clear why Spatafora et al. (58) observed SloC repression with as little as 1 μM Fe, whereas greater than 500 μM Fe was required to observe even partial repression in our hands. We initially suspected that the form of Fe employed was important, since Spatafora et al. used Fe^{3+}, whereas we used Fe^{2+}. Figure 2B indicates that Fe^{3+} also failed to repress SloC. In an attempt to resolve this difference, we also grew cells in the same media used by Spatafora et al.: FMC medium treated with Chelex 100 to remove metals and then supplemented with 80 μM Mg^{2+} and 0.4 μM Mn^{2+}. We observed very low levels of SloC expression whether

TABLE 2. Kinetic parameters for Fe uptake in wild-type and *slo* mutant strains

Strain	K_m^{Fe} (μM)	V_{max} (pmol/10^7 cells/min)
V403 (wild type)	9.7 ± 4.1	1.7 ± 0.2
V2613 (*sloC*)	83 ± 420	1.5 ± 6.9
JFP14 (*sloA*)	94 ± 409[a]	0.8 ± 3.1[a]
V2643 (*sloR*)	9.9 ± 1.7	8.7 ± 0.7

[a] Average ± standard error of two independent experiments. All other values are from three experiments.

TABLE 3. Virulence of *S. mutans* V403 and *sloA::aphA-3* mutants in a rat model of endocarditis

Strain	Genotype	No. of rats infected/total (% infected)[a]	*P* value
Expt 1			
V403	Wild type	5/11 (45)	0.0539[b]
JFP14	*sloA1::aphA-3*	0/7 (0)	
Expt 2			
V403	Wild type	2/4 (50)	0.4286[b]
JFP14	*sloA1::aphA-3*	0/4 (0)	
Combined			
V403	Wild type	7/15 (47)	0.0231[c]
JFP14	*sloA1::aphA-3*	0/11 (0)	

[a] Surgical survivors with correct catheter placement at necropsy.
[b] Fisher's exact probability test.
[c] Cochran-Mantel-Haenzel test.

Fe^{3+} was added to the medium or not (data not shown). We interpret these results as SloR-dependent repression of SloC effected by the 0.4 μM Mn in the medium. In a recent study, it was reported that residues D8 and E99 of *B. subtilis* MntR are responsible for the Mn selectivity of this SloR homolog (22). SloR also contains these residues (D7 and E99), in agreement with expectations if SloR is an Mn-responsive regulator.

We next attempted to determine whether the SloABC proteins function in Mn or Fe transport. Two lines of evidence suggest that these proteins transport both metals. First, the growth studies shown in Fig. 4 indicate that the *sloABC* genes are required for aerobic growth in the presence of Fe and low concentrations of Mn. This is most easily explained by SloABC-mediated transport of these metals. Although the reason for the poor and inconsistent growth of V2643 (*sloR*) in Fe^{3+} and of all strains in Fe^{2+} was not determined (Fig. 4B and C) (data not shown), this may be due to the difficulty of maintaining Fe in a soluble and available form for extended periods in an aerobic environment. Second, the ^{55}Fe uptake studies presented in Fig. 5A and Table 2 indicate that *sloA* and Δ*sloC* mutants acquire far less Fe than wild-type cells, and a *sloR* mutant acquires more. These results are consistent with SloABC-mediated uptake of Fe. Addition of Mn as a competitor increased the K_m^{app} for Fe in a dose-dependent fashion (Fig. 5B). Because the cells were exposed to Mn for only 2 min, the decrease in Fe acquisition cannot be due to Mn-dependent repression of the SloABC proteins. Instead, it is most likely that Mn competes with Fe for binding and transport and apparently does so with greater efficiency than Fe. When a substrate and an inhibitor are both acted upon by the same enzyme, the K_i of the inhibitor is equivalent to its K_m (12). The K_i for Mn was 2.0 μM, which is about one-fourth the K_m of Fe in wild-type *S. mutans*. Although the standard error for K_i was large, this suggests that Mn would be preferentially transported by the SloABC system, compared to Fe. These results can again be compared with those of Spatafora et al. (58). Using ^{55}Fe and ^{54}Mn uptake assays, Spatafora et al. reported that a *sloC* mutant was unaffected in Fe uptake, that a *sloR* mutant exhibited increased uptake, and that Mn uptake was not significantly affected by mutations in *sloC* or *sloR*. These results were interpreted as evidence of SloC-mediated Fe uptake. We also observed increased Fe uptake in a *sloR* mutant, but our results concerning a Δ*sloC* mutant and Mn uptake differ. One possible explanation for these differences is that our study measured uptake for 2 min, whereas Spatafora et al. grew cells overnight in the presence of radioisotopes. Longer incubations might allow for other transport systems to take up sufficient metal to reach equilibrium in both mutant and wild-type cells (58). It has been proposed that *S. gordonii* contains at least two Mn transporters in addition to the SloABC-homologous system: one of which is an NRAMP-like protein (27), and the other is a second ABC transport system encoded by the *adc* operon (40). An NRAMP-like gene also exists in the *S. mutans* genome (27), as does an *adc* operon, although *adcA* seems to encode a secreted protein in *S. mutans* (40). This could account for the aerobic growth of the *sloA* and Δ*sloC* mutants in Mn concentrations ≥1 μM. It may also account for the deviation of fit to the regression line shown in Fig. 5B, since competition for Fe uptake could be reduced by these additional Mn transport systems at the higher concentrations of Mn tested.

There is precedent for uptake of two or more metals by one ABC transport system. *Sca*CBA is a high-affinity Mn transport system that also transports Zn (36, 37). The *mtsABC* in *S. pyogenes* encodes an Fe/Zn transport system that also transports Mn (29, 30). The Adc Zn transporter in *S. pneumoniae* was proposed to transport Mn as well (14), and observations on the FimCBA system in *S. parasanguis* suggest that it also transports both Fe and Mn (11, 49). The regulatory mechanisms and the importance of these metal ion homeostasis systems are not yet fully elucidated. In *S. mutans*, the functional and regulatory properties of the *sloABCR* operon may provide unique benefits. As shown previously by others (44) and in our own studies (data not shown), *S. mutans* requires neither Fe nor Mn when grown anaerobically. In the presence of oxygen, either Fe or Mn is required, with Mn supporting growth that is faster and that reaches higher final densities (44) (Fig. 4). The growth-promoting effect of both metals likely results from their ability to serve as alternate cofactors for superoxide dismutase in *S. mutans* (43). Thus, the SloABC system may have evolved to take up either metal, because both have similar functions. Under aerobic conditions, however, free Fe can also generate toxic hydroxyl radicals by a Fenton-type reaction (8). Thus, Fe must be considered a "double-edged sword" for the cell, potentially affording both harm and protection. In contrast, free Mn does not induce the Fenton-type reaction (9) and has been demonstrated to act as an antioxidant in *Lactobacillus plantarum* (3) and *Neisseria gonorrhoeae* (64). It is therefore not surprising that Mn is more effective than Fe for supporting aerobic growth. Our findings suggest that the SloABC system preferentially transports Mn when it is present and that accumulation of Mn results in SloR-mediated down-regulation of the system, which would then prevent Fe uptake. This system would seem to ensure that Mn is taken up preferentially if both Mn and Fe are present but would still allow Fe uptake in the absence of Mn. This ability may be physiologically relevant in the oral environment. Chicharro et al. measured concentrations of both Mn and Fe in the saliva of 40 young men (10). Variability was high, but mean concentrations of 80 to 84 μM for Fe and 25 to 36 μM for Mn were reported. Because this analysis did not assess binding of these metals to lactoferrin and other salivary constituents, it is not clear what amount of either metal is available for uptake by *S. mutans*.

Finally, our study further clarifies the role of the SloABC proteins in the virulence of *S. mutans*. Our previous study indicated that the Δ*sloC* mutant, V2613, was indistinguishable from its wild-type parent in a rat model of caries (34). These results were confirmed by Spatafora et al. (58), who examined a *sloC* mutant. This group also examined a *sloR* mutant in the same model and found that it likewise retained virulence. In contrast to the caries model, we found that V2613 was avirulent in a rat endocarditis model. Possible explanations for this result included a nutritional deficiency resulting from loss of SloABC-mediated metal transport and reduced adherence due to loss of SloC. An adhesin function for SloC is suggested by its homology to the FimA protein of *S. parasanguis*. FimA has been shown to be a virulence factor for endocarditis and to be important for binding to fibrin monolayers, which serve as an in vitro model of a damaged heart valve (7). Because SloC may

possess both functions, it is impossible to determine by *sloC* mutation which function is required for virulence. Construction of a *sloA* mutant, JFP14, for this study was designed to address this question. In contrast to *sloC*, the *sloA* gene encodes the ATP-binding component of the SloABC uptake system and should have no function other than providing energy for transport (24). Furthermore, Fig. 3B indicates that JFP14 produces near-wild-type levels of SloC protein. If SloC-mediated adherence occurs, it should be intact in this mutant. Therefore, while this experiment does not address the possibility that SloC functions as an adhesin, the failure of JFP14 to cause endocarditis (Table 3) indicates that loss of metal transport is sufficient to account for loss of virulence in this model. The concentration of free Fe in the bloodstream is on the order of 10^{-24} M (6). It is therefore highly unlikely that Fe transport by the SloABC system is important for survival of *S. mutans* in the blood. In contrast, Mn levels in the bloodstream are reported to be about 1 μg or 0.02 μmol/liter, with a portion of this bound to serum proteins (38, 55). This concentration appears to be in the range that could allow growth of our wild-type strain but not the *sloA* or Δ*sloC* mutant (Fig. 4D). In support of this hypothesis, addition of 10, 20, 30, or 40% rat serum to BCMg810 medium resulted in aerobic growth of the wild-type and *sloR* mutant strains, but not the *sloA* and Δ*sloC* mutants (data not shown). Therefore, we conclude that loss of Mn transport is responsible for loss of endocarditis virulence in the *sloA* mutant.

These findings are encouraging in terms of vaccine development efforts based on the SloC homolog, FimA. Vaccination with FimA protects rats against endocarditis caused by *S. parasanguis* (66) *S. mitis*, *S. salivarius*, and *S. mutans* (35). If the LraI proteins in each of these species possess both the fibrin-binding properties of FimA and the Mn-transporting function of SloC, any mutation in the gene that results in loss of reactivity of the protein to anti-FimA antibodies may also result in loss of a function critical to pathogenesis.

ACKNOWLEDGMENTS

We thank Chris Shelton and Sherif Elhady for assistance in the creation and verification of the *sloA* mutant and Erin Grap for performing the rat serum growth studies. We thank Al Best, Darrell Peterson, Nick Jakubovics, Howard Jenkinson, and the Molecular Pathogenesis group at Virginia Commonwealth University for helpful discussions.

Support for this project was provided by PHS grant DE04224 to F. L. Macrina.

REFERENCES

1. **Al-Karaawi, Z. M., V. S. Lucas, M. Gelbier, and G. J. Roberts.** 2001. Dental procedures in children with severe congenital heart disease: a theoretical analysis of prophylaxis and non-prophylaxis procedures. Heart **85:**66–68.
2. **Alloing, G., M. C. Trombe, and J. P. Claverys.** 1990. The *ami* locus of the gram-positive bacterium *Streptococcus pneumoniae* is similar to binding protein-dependent transport operons of gram-negative bacteria. Mol. Microbiol. **4:**633–644.
3. **Archibald, F. S., and I. Fridovich.** 1981. Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. J. Bacteriol. **145:**442–451.
4. **Baddour, L. M.** 1999. Immunization for prevention of infective endocarditis. Curr. Infect. Dis. Rep. **1:**126–128.
5. **Bayliss, R., C. Clarke, C. Oakley, W. Somerville, and A. G. Whitfield.** 1983. The teeth and infective endocarditis. Br. Heart J. **50:**506–512.
6. **Braun, V., and H. Killmann.** 1999. Bacterial solutions to the iron-supply problem. Trends Biochem. Sci. **24:**104–109.
7. **Burnette-Curley, D., V. Wells, H. Viscount, C. L. Munro, J. C. Fenno, P. Fives-Taylor, and F. L. Macrina.** 1995. FimA, a major virulence factor associated with *Streptococcus parasanguis* endocarditis. Infect. Immun. **63:**4669–4674.
8. **Byers, B. R., and J. E. L. Arceneaux.** 1998. Microbial iron transport: iron assimilation by pathogenic microorganisms. Metal Ions Biol. Syst. **35:**37–65.
9. **Cheton, P. L., and A. F. S.** 1988. Manganese complexes and the generation and scavenging of hydroxyl free radicals. Free Radic. Biol. Med. **5:**325–333.
10. **Chicharro, J. L., V. Serrano, R. Urena, A. M. Gutierrez, A. Carvajal, P. Fernandez-Hernando, and A. Lucia.** 1999. Trace elements and electrolytes in human resting mixed saliva after exercise. Br. J. Sports Med. **33:**204–207.
11. **Claverys, J.** 2001. A new family of high-affinity ABC manganese and zinc permeases. Res. Microbiol. **152:**231–243.
12. **Cornish-Bowden, A.** 1979. Fundamentals of enzyme kinetics. Butterworths, London, United Kingdom.
13. **Dajani, A. S., K. A. Taubert, W. Wilson, A. F. Bolger, A. Bayer, P. Ferrieri, M. H. Gewitz, S. T. Shulman, S. Nouri, J. W. Newburger, C. Hutto, T. J. Pallasch, T. W. Gage, M. E. Levison, G. Peter, and G. Zuccaro, Jr.** 1997. Prevention of bacterial endocarditis—recommendations by the American Heart Association. JAMA **277:**1794–1801.
14. **Dintilhac, A., G. Alloing, C. Granadel, and J. P. Claverys.** 1997. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol. Microbiol. **25:**727–739.
15. **Dintilhac, A., and J. P. Claverys.** 1997. The *adc* locus, which affects competence for genetic transformation in *Streptococcus pneumoniae*, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. Res. Microbiol. **148:**119–131.
16. **Durack, D. T.** 1995. Prevention of infective endocarditis. N. Engl. J. Med. **332:**38–44.
17. **Evans, S. L., J. E. L. Arceneaux, B. R. Byers, M. E. Martin, and H. Aranha.** 1986. Ferrous iron transport in *Streptococcus mutans*. J. Bacteriol. **168:**1096–1099.
18. **Fenno, J. C., A. Shaikh, G. Spatafora, and P. Fives-Taylor.** 1995. The *fimA* locus of *Streptococcus parasanguis* encodes an ATP-binding membrane transport system. Mol. Microbiol. **15:**849–863.
19. **Franken, C., G. Haase, C. Brandt, J. Weber-Heynemann, S. Martin, C. Lammler, A. Podbielski, R. Lutticken, and B. Spellerberg.** 2001. Horizontal gene transfer and host specificity of beta-haemolytic streptococci: the role of a putative composite transposon containing *scpB* and *lmb*. Mol. Microbiol. **41:**925–935.
20. **Ganeshkumar, N., M. Song, and B. C. McBride.** 1988. Cloning of a *Streptococcus sanguis* adhesin which mediates binding to saliva-coated hydroxyapatite. Infect. Immun. **56:**1150–1157.
21. **Gilson, E., G. Alloing, T. Schmidt, J. P. Claverys, R. Dudler, and M. Hofnung.** 1988. Evidence for high affinity binding-protein dependent transport systems in gram-positive bacteria and in *Mycoplasma*. EMBO J. **7:**3971–3974.
22. **Guedon, E., and J. D. Helmann.** 2003. Origins of metal ion selectivity in the DtxR/MntR family of metalloregulators. Mol. Microbiol. **48:**495–506.
23. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:**331–384.
24. **Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gill, and M. P. Gallagher.** 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomembr. **22:**571–592.
25. **Horsburgh, M. J., S. J. Wharton, A. G. Cox, E. Ingham, S. Peacock, and S. J. Foster.** 2002. MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. Mol Microbiol. **44:**1269–1286.
26. **Horton, R. M.** 1995. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. Mol. Biotechnol. **3:**93–99.
27. **Jakubovics, N. S., and H. F. Jenkinson.** 2001. Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. Microbiology **147:**1709–1718.
28. **Jakubovics, N. S., A. W. Smith, and H. F. Jenkinson.** 2000. Expression of the virulence-related Sca (Mn^{2+}) permease in *Streptococcus gordonii* is regulated by a diphtheria toxin metallorepressor-like protein ScaR. Mol. Microbiol. **38:**140–153.
29. **Janulczyk, R., J. Pallon, and L. Bjorck.** 1999. Identification and characterization of a *Streptococcus pyogenes* ABC transporter with multiple specificity for metal cations. Mol. Microbiol. **34:**596–606.
30. **Janulczyk, R., S. Ricci, and L. Björck.** 2003. MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of *Streptococcus pyogenes*. Infect. Immun. **71:**2656–2664.
31. **Jenkinson, H. F.** 1994. Cell surface protein receptors in oral streptococci. FEMS Microbiol. Lett. **121:**133–140.
32. **Kehres, D. G., A. Janakiraman, J. M. Slauch, and M. E. Maguire.** 2002. Regulation of *Salmonella enterica* serovar Typhimurium *mntH* transcription by H_2O_2, Fe^{2+}, and Mn^{2+}. J. Bacteriol. **184:**3151–3158.
33. **Kiska, D. L., and F. L. Macrina.** 1994. Genetic analysis of fructan-hyperproducing strains of *Streptococcus mutans*. Infect. Immun. **62:**2679–2686.
34. **Kitten, T., C. L. Munro, S. M. Michalek, and F. L. Macrina.** 2000. Genetic characterization of a *Streptococcus mutans* LraI family operon and role in virulence. Infect. Immun. **68:**4441–4451.
35. **Kitten, T., C. L. Munro, A. Wang, and F. L. Macrina.** 2002. Vaccination with FimA from *Streptococcus parasanguis* protects rats from endocarditis caused by other viridans streptococci. Infect. Immun. **70:**422–425.

36. **Kolenbrander, P. E., R. N. Andersen, R. A. Baker, and H. F. Jenkinson.** 1998. The adhesion-associated *sca* operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn^{2+} uptake. J. Bacteriol. **180:**290–295.
37. **Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar.** 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. Infect. Immun. **62:**4469–4480.
38. **Krachler, M., E. Rossipal, and D. Micetic-Turk.** 1999. Concentrations of trace elements in sera of newborns, young infants, and adults. Biol. Trace Elem. Res. **68:**121–135.
39. **Lindler, L. E., and F. L. Macrina.** 1986. Characterization of genetic transformation in *Streptococcus mutans* by using a novel high-efficiency plasmid marker rescue system. J. Bacteriol. **166:**658–665.
40. **Loo, C. Y., K. Mitrakul, I. B. Voss, C. V. Hughes, and N. Ganeshkumar.** 2003. Involvement of the *adc* operon and manganese homeostasis in *Streptococcus gordonii* biofilm formation. J. Bacteriol. **185:**2887–2900.
41. **Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones.** 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25:**145–150.
42. **Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell.** 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene **19:**345–353.
43. **Martin, M. E., B. R. Byers, M. O. Olson, M. L. Salin, J. E. Arceneaux, and C. Tolbert.** 1986. A *Streptococcus mutans* superoxide dismutase that is active with either manganese or iron as a cofactor. J. Biol. Chem. **261:**9361–9367.
44. **Martin, M. E., R. C. Strachan, H. Aranha, S. L. Evans, M. L. Salin, B. Welch, J. E. L. Arceneaux, and B. R. Byers.** 1984. Oxygen toxicity in *Streptococcus mutans*: manganese, iron, and superoxide dismutase. J. Bacteriol. **159:**745–749.
45. **Michalek, S. M., J. R. McGhee, and J. M. Navia.** 1975. Virulence of *Streptococcus mutans*: a sensitive method for evaluating cariogenicity in young gnotobiotic rats. Infect. Immun. **12:**69–75.
46. **Munro, C., S. M. Michalek, and F. L. Macrina.** 1991. Cariogenicity of *Streptococcus mutans* V403 glucosyltransferase and fructosyltransferase mutants constructed by allelic exchange. Infect. Immun. **59:**2316–2323.
47. **Munro, C. L.** 1998. The rat model of endocarditis. Methods Cell Sci. **20:** 203–207.
48. **Munro, C. L., and F. L. Macrina.** 1993. Sucrose-derived exopolysaccharides of *Streptococcus mutans* V403 contribute to infectivity in endocarditis. Mol. Microbiol. **8:**133–142.
49. **Oetjen, J., P. Fives-Taylor, and E. H. Froeliger.** 2002. 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. Infect. Immun. **70:**5706–5714.
50. **Oligino, L., and P. Fives-Taylor.** 1993. Overexpression and purification of a fimbria-associated adhesin of *Streptococcus parasanguis*. Infect. Immun. **61:** 1016–1022.
51. **Patzer, S. I., and K. Hantke.** 2001. Dual repression by Fe^{2+}-Fur and Mn^{2+}-MntR of the *mntH* gene, encoding an NRAMP-like Mn^{2+} transporter in *Escherichia coli*. J. Bacteriol. **183:**4806–4813.
52. **Posey, J. E., J. M. Hardham, S. J. Norris, and F. C. Gherardini.** 1999. Characterization of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum*. Proc. Natl. Acad. Sci. USA **96:**10887–10892.
53. **Que, Q., and J. D. Helmann.** 2000. Manganese homeostasis in *Bacillus subtilis* is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol. Microbiol. **35:**1454–1468.
54. **Roberts, G. J.** 1999. Dentists are innocent! "Everyday" bacteremia is the real culprit: a review and assessment of the evidence that dental surgical procedures are a principal cause of bacterial endocarditis in children. Pediatr. Cardiol. **20:**317–325.
55. **Scheuhammer, A. M., and M. G. Cherian.** 1985. Binding of manganese in human and rat plasma. Biochim. Biophys. Acta **840:**163–169.
56. **Schroeder, V. A., S. M. Michalek, and F. L. Macrina.** 1989. Biochemical characterization and evaluation of virulence of a fructosyltransferase-deficient mutant of *Streptococcus mutans* V403. Infect. Immun. **57:**3560–3569.
57. **Seymour, R. A., R. Lowry, J. M. Whitworth, and M. V. Martin.** 2000. Infective endocarditis, dentistry and antibiotic prophylaxis: time for a rethink? Br. Dent. J. **189:**610–616.
58. **Spatafora, G., M. Moore, S. Landgren, E. Stonehouse, and S. Michalek.** 2001. Expression of *Streptococcus mutans fimA* is iron-responsive and regulated by a DtxR homologue. Microbiology **147:**1599–1610.
59. **Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, N. Schnitzler, R. Lütticken, and A. Podbielski.** 1999. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. Infect. Immun. **67:**871–878.
60. **Strom, B. L., E. Abrutyn, J. A. Berlin, J. L. Kinman, R. S. Feldman, P. D. Stolley, M. E. Levison, O. M. Korzeniowski, and D. Kaye.** 1998. Dental and cardiac risk factors for infective endocarditis—a population-based, case-control study. Ann. Intern. Med. **129:**761–769.
61. **Sutcliffe, I. C., and R. R. Russell.** 1995. Lipoproteins of gram-positive bacteria. J. Bacteriol. **177:**1123–1128.
62. **Tartof, K. D., and C. A. Hobbs.** 1987. Improved media for growing plasmid and cosmid clones. Focus **9:**12.
63. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11:**649–655.
64. **Tseng, H. J., Y. Srikhanta, A. G. McEwan, and M. P. Jennings.** 2001. Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. Mol. Microbiol. **40:**1175–1186.
65. **van der Meer, J. T., W. van Vianen, E. Hu, W. B. van Leeuwen, H. A. Valkenburg, J. Thompson, and M. F. Michel.** 1991. Distribution, antibiotic susceptibility and tolerance of bacterial isolates in culture-positive cases of endocarditis in The Netherlands. Eur. J. Clin. Microbiol. Infect. Dis. **10:**728–734.
66. **Viscount, H. B., C. L. Munro, D. Burnette-Curley, D. L. Peterson, and F. L. Macrina.** 1997. Immunization with FimA protects against *Streptococcus parasanguis* endocarditis in rats. Infect. Immun. **65:**994–1002.