Expression of *Streptococcus mutans* *fimA* is iron-responsive and regulated by a DtxR homologue

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Iron uptake, transport and storage in *Streptococcus mutans*, the principal causative agent of human dental cavities, is unexplored despite early reports in the literature which predict a role for this trace metal in cariogenesis. Experiments in the authors' laboratory revealed several iron-responsive proteins in *S. mutans*, one of which reacted with a polyclonal antiserum directed against the FimA fimbrial adhesin from *Streptococcus parasanguis* on Western blots. The results of Western blot and Northern hybridization experiments support an inverse relationship between iron availability and *S. mutans* *fimA* expression, and metal ion uptake experiments implicate FimA in *S. mutans* ⁵⁵Fe transport. Cloning of the *S. mutans* *fimA* homologue facilitated the construction of a *fimA* knockout mutant which grew poorly in an iron-limiting medium relative to the wild-type progenitor strain, lending further support to a role for FimA in *S. mutans* iron transport. The authors also identified and cloned a *dtxR*-like gene (*dlg*) located downstream of *fimA* on the *S. mutans* chromosome, and noted increased *fimA* expression in a *S. mutans* *dlg* knockout mutant relative to wild-type on RNA spot blots and Western blots. The uptake of ⁵⁵Fe, which was also significantly increased in this mutant, was compromised in a *fimA/dlg* double knockout. These findings are consistent with a role for Dlg in the iron-mediated regulation of *fimA*, and possibly other *S. mutans* iron transporters. Finally, the cariogenic potential of the *fimA* and *dlg* knockout mutants was not significantly different from that of the wild-type progenitor in a germ-free rat model.

Keywords: oral streptococci, *fimA*, *dtxR*, regulation, metals, iron uptake

INTRODUCTION

Elemental iron is an essential micronutrient for most bacterial pathogens. In the human host, 99.9% of intracellular iron is bound to ferritin storage proteins or haem compounds (Weinberg, 1978), while extracellular iron is complexed to the carrier proteins transferrin (in serum) and lactoferrin (in mucosal secretions) (Finkelstein *et al.*, 1983). The concentration of free iron in living tissues is only 10⁻¹² µM, however, well below the needs of invading pathogens (Weinberg, 1978). Thus, bacteria experience a shift from high to low iron availability upon entering a mammalian host, which can signal the production of iron-chelating siderophores

and/or the synthesis of iron-specific receptors and transport proteins (Wooldridge & Williams, 1993). In addition, iron limitation can trigger virulence gene expression in invading pathogens.

Despite a well-documented role for iron in promoting the pathogenesis of some Gram-negative microorganisms (Bullen *et al.*, 1978), the involvement of this micronutrient in disease brought on by Gram-positive bacteria has not been extensively investigated. While early epidemiological and clinical studies suggested that certain trace metals in food and drinking water, including iron, may be associated with the development of dental caries (Adkins & Losee, 1970; Aranha *et al.*, 1982), the mechanism(s) by which iron exerts its putative effect(s) on *Streptococcus mutans*-induced cariogenesis remain unclear. In previous work, the absence of typical

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Abbreviation: IR, inverted repeat.

hydroxamate- and/or catecholate-like siderophores from *S. mutans* was suggested by biochemical assays specific for these compounds (Evans *et al.*, 1986). In our laboratory, dialysis bag experiments (Husson *et al.*, 1993) conducted in parallel with a universal siderophore detection assay (Schwyn & Neilands, 1987) have confirmed that *S. mutans* does not elaborate these small iron-chelating molecules (unpublished observations).

FimA is a 36 kDa fimbrial lipoprotein adhesin on the surface of *Streptococcus parasanguis* (also known as *S. parasanguinis*), the primary colonizer of dental plaque and major player in subacute endocarditis (Burnette-Curley *et al.*, 1995; Viscount *et al.*, 1997). Interestingly, reports in the literature implicate a role for *S. parasanguis* FimA in adherence to fibrin (Burnette-Curley *et al.*, 1995) but not to saliva-coated hydroxyapatite (Froeliger & Fives-Taylor, 2000). In the present study, we identified a 34 kDa *fimA* homologue in *S. mutans*.

FimA belongs to the lipoprotein receptor antigen I (LraI) family (Fenno *et al.*, 1995) of proteins, which is transcribed as part of ABC-transporter-type operons. Reports in the literature indicate that in addition to functioning as adhesins, LraI proteins are also involved in metal ion transport (Dintilhac & Claverys, 1997; Kolenbrander *et al.*, 1998). Among the LraI adhesins previously described in the streptococci are ScaA in *S. gordonii* (Kolenbrander *et al.*, 1994), PsaA and AdcA in *S. pneumoniae* (Dintilhac *et al.*, 1997), ScbA in *S. crista* (Correia *et al.*, 1996), SsaB in *S. sanguis* (also known as *S. sanguinis*) (Ganeshkumar *et al.*, 1991), Lmb in *S. agalactiae* (Spellerberg *et al.*, 1998) and FimA in *S. parasanguis* (Burnette-Curley *et al.*, 1995; Fenno *et al.*, 1995). Of these adhesins, ScaA and PsaA are known to facilitate the transport of manganese ions (Kolenbrander *et al.*, 1998; Dintilhac *et al.*, 1997) while AdcA is reported to be a putative transporter of zinc (Dintilhac *et al.*, 1997). Other studies describe the translocation of metal ions, including iron, by an ABC transporter lipoprotein in the group A streptococci (Janulcyk *et al.*, 1999). In the present study, we generated a knockout mutation in the *S. mutans fimA* gene to define a putative role for the FimA lipoprotein in iron uptake/transport.

The regulation of iron uptake/transport by the *fur* gene product is well documented in Gram-negative bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae* and *Vibrio cholerae* (Zimmerman *et al.*, 1984; Fleming *et al.*, 1983; Genco & Desai, 1996; Butteron *et al.*, 1992), in which Fur-Fe²⁺ complexes bind to the bacterial chromosome and prevent transcription of iron-dependent genes. Homologues of *fur* have not been identified in *S. mutans*. Rather, in the present study we identified a *dtxR*-like gene (*dlg*) downstream of the *fimA* operon on the *S. mutans* chromosome for which there is a homologue in *Corynebacterium diphtheriae*. DtxR in *C. diphtheriae* is an iron-dependent metalloregulatory protein that complexes with Fe²⁺ to regulate the expression of diphtheria toxin and other virulence-associated genes (Boyd *et al.*, 1990; Hennecke, 1990; Schmidt, 1997; Schiering *et al.*, 1995). Herein, we present evidence that is consistent

with a role for the *S. mutans* DtxR homologue in iron-dependent regulation of *S. mutans fimA* expression.

METHODS

Media preparation. A chemically defined FMC medium (Terleckyj *et al.*, 1975) was prepared without magnesium, manganese or iron as previously described (Spatafora & Moore, 1998). Briefly, the medium was stirred vigorously over 30 g l⁻¹ Chelex-100 chelating resin (Supelco) for 60 min at 4 °C to remove trace metals (the resin was pre-treated to remove fines and residual iminodiacetic acid moieties as described by the manufacturer). The medium was then filter-sterilized and amended with MgSO₄ (80 µM), MnSO₄ (0.4 µM) and ferric citrate (1.2 µM) to yield an iron-replete medium. Alternatively, the Chelex-treated medium was supplemented with only MgSO₄ and MnSO₄ as described above to generate an iron-depleted environment. For expression studies, ferric citrate was added to the iron-depleted medium described above at concentrations ranging from 0.01 to 10 µM. Absolute metal ion concentrations were confirmed on a Thermo Jarrell Ash Inductively Coupled Argon Plasma (ICAP 61) analyser (Coish & Sinton, 1992) that had been previously standardized with a metal solution containing 1000 p.p.m. iron, magnesium and manganese.

Bacterial culture conditions. Wild-type *S. mutans* UA130 (serotype *c*) was grown overnight at 37 °C and 5% CO₂ in 14 ml Todd-Hewitt broth (THB). *S. mutans* GMS700 and GMS800 were grown overnight as described in THB supplemented with kanamycin (250 µg ml⁻¹), and GMS850 was grown in THB supplemented with erythromycin (10 µg ml⁻¹). Cultures were stored in THB containing 20% (v/v) sterile glycerol at -80 °C.

For growth-curve determinations and Northern hybridization experiments, *S. mutans* was grown at 37 °C with 5% CO₂ as described, and the cells harvested by centrifugation at 7000 r.p.m. for 10 min in an SS34 rotor. The cell pellets were washed three times in iron-depleted FMC and resuspended in 1 ml of the same. Fourteen millilitres of FMC containing various concentrations of iron were then inoculated with 150 µl of this cell concentrate and grown as described for up to 24 h.

Total RNA for spot blotting was derived from overnight cultures of *S. mutans* UA130 and GMS800 grown in THB or THB supplemented with kanamycin, respectively. These cultures were used to inoculate 45 ml THB and grown to early-, mid- and late-exponential and stationary phases as described.

Protein isolation and SDS-PAGE. For the preparation of cell lysates, 14 ml volumes of FMC containing 0.01–10 µM ferric citrate were inoculated, and the cells grown and harvested as described. The cell pellets were resuspended in cold phosphate-buffered saline containing 0.5 mM PMSF and the cell suspensions transferred to microcentrifuge tubes containing 1/3 vol. zirconium beads (0.1 mm). The cells were disrupted in a Mini-Bead Beater (Biospec) at 4 °C for 3 min and cellular debris removed by low-speed centrifugation for 1 min in a refrigerated microcentrifuge. Total protein determinations were performed for each sample using a BCA protein assay kit (Pierce) and bovine serum albumin as a standard. The cell lysates were combined with an equal volume of 2 × sample buffer (Laemmli, 1970) and incubated at room temperature for 2 h prior to loading onto a 5–15% SDS-PAGE gradient gel. Electrophoresis proceeded at 90 V for 16 h, followed by staining with Coomassie brilliant blue R-250 (Sigma) and/or silver stain (AMRESCO).

Table 1. Plasmids

Plasmid	Phenotype	Source*
pBR322 Ω Km-2	4.3 kb pBR322 with 2.0 kb Ω Km-2 cassette, Ap ^R Tc ^R Km ^R	M. Caperon
pfimEm1	pMM1 with an Em ^R gene inserted in the middle of the <i>fimA</i> gene, 5.4 kb, Em ^R Ap ^R	This laboratory
pfimEm2	4.3 kb pfimEm1 with Δ Ap ^R , Em ^R Ap ^S	This laboratory
pGEMT-EZ	3.0 kb TA cloning vector, Ap ^R	Promega
pMM1	pGEMT-EZ vector with 1.5 kb amplicon harbouring 5' end of <i>S. mutans</i> <i>fimA</i> gene, Ap ^R	This laboratory
pMM2	pMM1 with Ω Km-2 cloned into the <i>fimA</i> coding sequence, 6.6 kb, Ap ^R Km ^R	This laboratory
pMM3	pMM2 with Δ Ap ^R , 5.5 kb, Km ^R Ap ^S	This laboratory
pSG236	pVA838-derived, 10.26 kb, Em ^R	S. Goodman
pSL1	pGEMT-EZ with 1.4 kb amplicon harbouring the <i>S. mutans</i> <i>dlg</i> gene, Ap ^R	This laboratory
pSL2	pSL1 with <i>aphA3</i> in <i>dlg</i> coding sequence, 5.9 kb, Ap ^R Km ^R	This laboratory
pSL3	pSL2 with Δ Ap ^R , 4.8 kb, Km ^R Ap ^S	This laboratory
pVT389	6.0 kb pET27b with Ω Km-2 inserted into <i>S. parasanguis</i> <i>fap1</i> gene	P. Fives-Taylor

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Western blotting. *S. mutans* proteins (50 μ g per lane) were resolved on each of two SDS-PAGE gels, one of which was stained with Coomassie blue. Proteins from the other gel were transferred electrophoretically to nitrocellulose membranes in a Hoeffer Western transfer apparatus at 0.2 A and 4 °C overnight. The membrane was blocked in 100 ml 5% (w/v) powdered skim milk for 1 h with gentle agitation, washed twice in 100 ml Tris-buffered saline/Tween for 10 min each, and then allowed to react with a 1:5000 dilution of a polyclonal rabbit antiserum directed against FimA from *S. parasanguis* (provided by Dr Paula Fives-Taylor, University of Vermont). Following three additional washes as described, a 1:10000 dilution of a horseradish-peroxidase-conjugated goat anti-rabbit antiserum (Sigma or Pierce) was applied. After several more washes, the blots were prepared for visualization by enhanced chemiluminescence (ECL) according to the manufacturer's recommendations (Amersham Life Science).

Cloning of the *S. mutans* *fimA* gene and construction of a *fimA* knockout mutation. The primers and plasmids used to clone and subsequently disrupt the *S. mutans* *fimA* gene are shown in Tables 1 and 2. The 5' end of the *S. mutans* *fimA* gene was amplified with primers SmfimA-F and SmfimA-R using sequence information derived from the GenBank database (accession number AF232688) and cloned into pGEMT-EZ (Promega) according to the recommendations of the supplier. The resulting recombinant, pMM1, includes a unique *Hind*III site 13 bp downstream of the *fimA* initiation codon which we exploited in subsequent knockout mutagenesis experiments. Specifically, a 2.0 kb Ω Km-2 kanamycin-resistance cassette (*aphA3* Ω) from plasmid pBR322Km Ω was cloned into the *Hind*III site within the *fimA* coding sequence on pMM1, and *E. coli* transformants harbouring the *fimA* knockout mutation were selected on L-agar supplemented with kanamycin (50 μ g ml⁻¹). The knockout mutation was confirmed by sequence analysis of plasmid DNA isolated and

purified on mini-prep spin columns (Qiagen). We disrupted the ampicillin-resistance gene resident on the resulting pMM2 construct with *Pvu*I, and recircularized the 5.5 kb fragment with T₄ DNA ligase (Promega) following gel extraction (Qiagen). Plasmid DNA (pMM3) was isolated from *E. coli* transformants demonstrating resistance to kanamycin, and confirmed by restriction enzyme mapping. To confirm ampicillin sensitivity, transformants were also replica-plated onto L-agar supplemented with 50 μ g ampicillin ml⁻¹. Finally, pMM3 was moved into *S. mutans* by electroporation (Spatafora *et al.*, 1995), and the double crossover event selected for on Todd-Hewitt agar (TH agar) plates supplemented with kanamycin (250 μ g ml⁻¹). The resulting *S. mutans* knockout mutant (GMS700) was confirmed by Southern blot analysis of restricted chromosomal DNA isolated as described by Sambrook *et al.* (1989). A DNA fragment internal to the *fimA* coding sequence was generated using primers fimA.pb-F and fimA.pb-R and radiolabelled for hybridization experiments.

Cloning of the *S. mutans* *dlg* gene and construction of a *dlg* knockout mutation. The primers and plasmids used to clone and disrupt the *S. mutans* *dlg* gene are shown in Tables 1 and 2. The *S. mutans* *dlg* gene was amplified with primers Sm-dlg-F and Sm-dlg-R using sequence information derived from the GenBank database (accession number AF232688) and cloned as a 1.4 kb amplicon into pGEMT-EZ (Promega) according to the recommendations of the supplier. A unique *Bst*Z17-I site located 456 bp downstream of the *dlg* start codon on the resulting recombinant, pSL1, served as the insertion site for a 1.5 kb *aphA3* kanamycin-resistance cassette. The cassette was amplified from plasmid pVT389 using primers aphA3-BstZ17I-F and aphA3-BstZ17I-R, and the resulting amplicon was digested with *Bst*Z17I to generate blunt ends. The cassette was then ligated into the *dlg* coding region, and *E. coli* transformants harbouring the *dlg* knockout mutation were selected on L-agar plates supplemented with kanamycin

Table 2. Primers

Restriction sites are underlined.

Primer	Sequence (5'–3')
SmFimA-F	GCAGCGGGTTCAAGCATTGTC
SmFimA-R	CAGCTGGGAGTAGGCGGAAACC
fimA.pb-F	CGCTGGTAATAAGGTTGTC
fimA.pb-R	CATTGTGACAGACCATTTCG
Sm-dlg-F	GGCTTACGATATTCCTTCT
Sm-dlg-R	GTTCGTGAATTCGTGGC
Sm-dlg-pb-F	CTGCTCCGGCTGTCTCAG
Sm-dlg-pb-R	CAGTGGCAACTGCTGATGT
aphA3-BstZ171-F	CAGCGAGTATACACCTGCCAGTCACGACGTTGTAAAA
aphA3-BstZ171-R	GGGGTGGTATACGGCAGCGGATAACAATTTACACAGG
ermAMHin-F	CCCAAGCTTGGGGCGGAAACGTAAAAGAAG
ermAMHin-R	CCCAAGCTTGGGGGAAGCTGTGTCAGTAGTATACC
rpsL-F	GAATGTAGATGCCTACAATTAACCA
rpsL-R	TTTACGACTCATTCTCTTTATCCC

(50 µg ml⁻¹). The resulting recombinant, pSL2, was isolated and digested with *PvuI* as described to disrupt ampicillin resistance, and a 4.7 kb fragment was gel-purified (Qiagen) and recircularized with T₄ DNA ligase (Promega). The final construct, pSL3, was isolated from *E. coli* transformants demonstrating kanamycin-resistance and sensitivity to ampicillin according to Qiagen protocols, and confirmed by restriction enzyme mapping. Plasmid pSL3 was then electroporated into *S. mutans*, and the *dlg* knockout mutation on the chromosome selected for on TH agar containing 250 µg kanamycin ml⁻¹. The double-crossover event was confirmed by PCR and Southern blot analysis, and the resulting mutant was called GMS800.

Construction of GMS850, a *S. mutans* fimA/dlg double knockout mutant. Primers ermAMHin-F and ermAMHin-R (Table 2) were used to amplify an erythromycin-resistance determinant (*ermAM*) from plasmid pSG236 (Goodman & Gao, 2000). The resulting 0.94 kb amplicon was digested with *HindIII* and ligated into the *HindIII* site located within the *fimA* coding sequence cloned on plasmid pMM1. *E. coli* transformants were selected on L-agar supplemented with 300 µg erythromycin ml⁻¹ from which putative recombinants (pfimEm1) were isolated and purified on Qiagen spin columns. The pfimEm1 construct was confirmed by restriction mapping, and digested with *PvuI* to disrupt the ampicillin-resistance gene on the plasmid. The resulting construct, pfimEm2, was subsequently used to transform the *S. mutans* *dlg* knockout mutant (GMS800) as described. Streptococcal transformants demonstrating resistance to both erythromycin (10 µg ml⁻¹) and kanamycin (250 µg ml⁻¹) on TH agar plates were selected for Southern blot analysis to confirm knockout mutations in the *fimA* and *dlg* genes on the *S. mutans* chromosome (data not shown).

DNA isolation. Chromosomal DNA was isolated from *S. mutans* UA130, GMS700, GMS800 and GMS850 using a modification of the method of Marmur (1961). The DNA was purified by caesium chloride/ethidium bromide equilibrium density-gradient centrifugation and digested with restriction enzymes (Promega) according to the recommendations of the supplier. Plasmid DNA was extracted and purified from *E. coli* transformants using Qiagen spin columns (Qiagen).

PCR. Primers used to generate amplicons for cloning and/or nick translation are shown in Table 2. Amplification with Red Taq polymerase (Sigma) was performed in a Hybaid PCR Express thermocycler with the following cycling conditions: 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min repeated for 35 cycles, followed by a 72 °C extension for 10 min.

Southern blotting. Restricted chromosomal DNAs from *S. mutans* UA130, GMS700 and GMS850, or from PCR products generated as described above, were resolved on 0.8 % agarose gels and transferred to nitrocellulose membranes according to the method of Southern (1975). The DNAs were cross-linked to the membranes in a FisherBrand cross-linker (FB-UVXL-1000) and probed with a 770 bp *fimA*-specific or a 500 bp *dlg*-specific amplicon that had been previously radiolabelled with [³²P]dATP by nick translation (Rigby *et al.*, 1977). Primers fimA.pb-F and fimA.pb-R or Sm-dlg-pb-F and Sm-dlg-pb-R (Table 2) were used to amplify the *fimA*- or *dlg*-specific probes, respectively. Filters were hybridized in 1 % BSA, 300 mM sodium phosphate, 7 % SDS and 100 mM EDTA, pH 8, at 60 °C for 16 h in a FisherBrand hybridization oven with gentle agitation. The membranes were then washed once for 10 min at 60 °C in 0.5 % BSA, 40 mM sodium phosphate, 5 % SDS and 1 mM EDTA, and twice for 10 min each at 60 °C in 40 mM sodium phosphate, 1 % SDS and 1 mM EDTA. Autoradiography (Kodak BIOMAX ML film) proceeded for up to 24 h at -80 °C in the presence of an intensifying screen.

RNA isolation. For Northern hybridization experiments, total intact RNA was isolated from *S. mutans* UA130 cultures grown as described in FMC supplemented with 0.01–10 µM ferric citrate. The cultures were centrifuged at 4 °C and 6000 r.p.m. in an SS34 rotor for 5 min and the cell pellets resuspended in 4 ml ice-cold sterile Tris/EDTA (TE) buffer, pH 8.0. To the cell suspensions, 3.5 g sterile, chilled, acid-washed glass beads (Sigma, 150–220 µm diameter) were added and immediately mixed with 3 ml phenol/chloroform/isoamyl alcohol (PCI), pH 4.3 (Sigma). After the addition of 0.25 ml 10 % SDS, the mixture was vortexed for 4 min with intermittent cooling on ice. The cell mixture was then centrifuged as described above and the aqueous phase extracted three more times with PCI, pH 4.3. Nucleic acid was

precipitated overnight at -20°C in the presence of 0.1 vol. 10 M LiCl and 2 vols ethanol. The RNA was pelleted by centrifugation at 8000 r.p.m. and 4°C in an SS34 rotor for 15 min and subsequently washed in ice-cold 70% ethanol. Finally, the RNA pellets were air-dried, resuspended in 50 μl sterile diethyl pyrocarbonate-treated water, and stored frozen at -80°C . For spot blot hybridization studies, total RNA was isolated from *S. mutans* by disruption in a reciprocating shaking device (FastPrep, Bio101) according to the Bio101 Fast RNA Blue protocol.

Northern and spot blots. Total RNA isolated from UA130 and GMS700 was resolved on a 0.8% formaldehyde agarose gel, transferred to a nitrocellulose membrane, and cross-linked in a FisherBrand cross-linker. Spot blots of total RNA isolated from GMS800 were prepared using a Schleicher and Schuell Minifold I apparatus according to the recommendations of the supplier. RNAs were probed with a radio-labelled 770 bp amplicon that is internal to the *fimA* coding sequence, or (as an internal control) with a 500 bp *rpsL* amplicon derived from the *S. mutans* chromosome using primers *rpsL*-F and *rpsL*-R (Table 2). Hybridization and wash conditions were as described for Southern blotting.

Metal ion uptake. *S. mutans* UA130, GMS700, GMS800 and GMS850 were grown overnight in THB with 250 μg kanamycin ml^{-1} or 10 μg erythromycin ml^{-1} when appropriate. The precultures (250 μl) were used to inoculate 50 ml prewarmed THB and grown to early exponential phase. Then 7.4×10^4 Bq $^{55}\text{FeCl}_3$ (3 μM) or $^{54}\text{MnCl}_2$ (0.03 μM) was added to 1 ml cells and the cultures were grown overnight as described. The bacteria were pelleted and washed three times in fresh THB, and radioactivity was measured in a scintillation counter calibrated for ^{55}Fe or ^{54}Mn . Control cultures grown in parallel and in the absence of radioisotope were serially diluted and plated on TH agar plates for bacterial enumeration.

Cariogenic potential of *S. mutans* GMS700 and GMS800 in germ-free rats. The cariogenic potential of *S. mutans* UA130, GMS700 and GMS800 was determined in young gnotobiotic Fischer rats. Nineteen-day-old weanling rats were challenged orally with approximately 10^8 c.f.u. ml^{-1} of the appropriate test strain. Animals were maintained on a sterile caries-promoting diet containing 5% sucrose (Michalek *et al.*, 1975) provided *ad libitum*. Colonization was assessed 2 d post-challenge and then weekly for the duration of the experiment by collecting faecal swab samples and culturing them on Mitis Salivarius (MS) agar (Difco) with or without kanamycin. Rats killed 35 d post-challenge were scored for caries (Keyes, 1958), and plaque microbiology was assessed on MS agar with appropriate selection to confirm the presence of UA130, GMS700 or GMS800.

Statistical analysis. Means and standard errors for caries scores and bacterial metal ion uptake were evaluated by analysis of variance using the Duncan and Kruskal-Wallis tests, respectively. Differences were considered to be significant at $P \leq 0.05$.

RESULTS

S. mutans UA130 requires iron for optimal growth

We monitored the growth of *S. mutans* UA130 in ferric citrate-depleted vs replete FMC medium and noted that growth was compromised in the former (Fig. 1). Similar results were obtained from *S. mutans* cultures grown in Chelex-treated FMC supplemented with ferric chloride

Fig. 1. *S. mutans* requires iron for optimal growth. *S. mutans* UA130 was grown for up to 24 h in Chelex-treated FMC containing 1.2 μM (filled triangles) or 0.4 μM (open triangles) ferric citrate prepared as described in Methods. The data indicate that *S. mutans* requires iron for optimal growth. OD₅₄₀ measurements were obtained in a Genosys 2 spectrophotometer. Growth curve experiments were performed in triplicate. The results of a single representative experiment are shown.

Fig. 2. Expression of *S. mutans* FimA is iron responsive. *S. mutans* proteins (50 μg per lane) isolated from UA130 cultures grown in FMC containing 0.01–10 μM ferric citrate and resolved on a 12.5% SDS-PAGE gel were reacted on a Western blot with a polyclonal antiserum directed against the 36 kDa FimA lipoprotein from *S. parasanguis*. A 34 kDa FimA protein reacted with this antiserum, the expression of which decreased as iron concentration increased. A secondary antiserum (Sigma) that cross-reacts non-specifically with a constitutively expressed protein of 48 kDa was applied in this experiment to provide an internal control against which *fimA* expression could be quantified (see Table 3).

(data not shown). Together these findings support iron as an essential micronutrient for the optimal growth of *S. mutans*.

SDS-PAGE and Western blot analysis of *S. mutans* UA130

Protein profiles derived from *S. mutans* UA130 whole cells grown in FMC supplemented with 0.01–10 μM ferric citrate revealed decreased expression of several proteins with increasing iron concentration (unpublished observations). In particular, immunoblots of *S. mutans* whole-cell lysates reacted with a polyclonal

Table 3. Effect of ferric citrate on *S. mutans* FimA expression

Ferric citrate concn (μM)	Absorbance units*		
	FimA	Control	Ratio†
0.01	1.6	2.6	0.62
0.1	2.6	2.8	0.93
1.0	0.85	2.2	0.39
10	0.15	2.3	0.07

* Measured on Western blots (see Fig. 2) with an LKB ULTRASCAN XL laser densitometer.

† Ratio of FimA to control protein expression.

antiserum directed against the *S. parasanguis* FimA lipoprotein revealed a 10-fold decrease in expression for the 34 kDa *S. mutans* FimA homologue as ferric citrate concentrations increased (see Fig. 2 and Table 3).

Northern blot analysis

To determine whether iron-responsive FimA expression in *S. mutans* is regulated at the level of transcription, we isolated total RNA from UA130 grown in Chelex-treated FMC containing 0.01 μM or 10 μM ferric citrate. The RNAs were resolved on 0.8% formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized with a 770 bp *fimA*-specific amplicon

which is internal to the *S. mutans fimA* coding region. The resulting Northern blot revealed decreased expression of the *S. mutans fimA* gene at the higher iron concentration (data not shown).

Cloning of the *S. mutans fimA* gene and confirmation of a *fimA* knockout mutation in *S. mutans* GMS700

The strategy used to clone and interrupt the *S. mutans fimA* coding sequence in *E. coli* is illustrated in Fig. 3(a). The $\Omega\text{Km-2}$ cassette (*aphA3* Ω) resident on pMM3 was then used to disrupt the wild-type *fimA* gene on the *S. mutans* UA130 chromosome by allelic exchange. The resulting knockout mutation in GMS700 was confirmed by Southern blot using a 770 bp *fimA*-specific probe. Insertion of the 1.8 kb $\Omega\text{Km-2}$ cassette into the *fimA* coding sequence on the GMS700 chromosome is supported by a shift in the *fimA*-containing DNA fragment from 2.6 kb in UA130 to 4.4 kb in GMS700 (Fig. 3b).

Cloning of the *S. mutans dlG* gene and confirmation of a *dlG* knockout mutation in *S. mutans* GMS800

The strategy used to clone and disrupt the *S. mutans dlG* gene in *E. coli* is illustrated in Fig. 4(a). A non-polar *aphA3* kanamycin-resistance cassette resident on pSL-3 was used to interrupt the wild-type *dlG* gene on the *S. mutans* UA130 chromosome by allelic exchange. The resulting knockout mutation in GMS800 was confirmed by PCR using *dlG*-specific primers, and then by Southern

Fig. 3. (a) Strategy used to construct a knockout mutation in the cloned *S. mutans fimA* gene. (b) Confirmation of the *fimA* knockout mutation in *S. mutans* GMS700. Left panel: chromosomal DNA isolated from UA130 and GMS700 was digested with *EcoRI* and resolved on an 0.8% agarose gel. Right panel: the restricted DNA was transferred to nitrocellulose, where it was probed for *fimA* with a 770 bp radiolabelled amplicon. The DNA fragment that hybridizes to the *fimA*-specific probe is 1.8 kb larger in GMS700 than in UA130. This is due to the insertion of the $\Omega\text{Km-2}$ kanamycin-resistance cassette (*aphA3* Ω) at the *fimA* locus in GMS700.

Fig. 4. (a) Strategy used to construct a knockout mutation in the cloned *S. mutans* *dlg* gene. (b) Confirmation of the *dlg* knockout mutation in *S. mutans* GMS800. Left panel: the *dlg* gene was PCR-amplified from UA130 and GMS800 using *dlg*-specific primers, resolved on a 0.8% agarose gel and stained with ethidium bromide. The amplicon derived from GMS800 is 1.5 kb larger than that derived from UA130, owing to the successful insertion of an *aphA3* kanamycin-resistance cassette. Right panel: the DNA from the PCR gel was transferred to nitrocellulose and probed for *dlg* with a 500 bp radiolabelled amplicon internal to the coding region of the gene. The DNA fragment that hybridizes to the *dlg*-specific probe is 1.5 kb larger in GMS800 than in UA130, owing to the insertion of the *aphA3* kanamycin-resistance cassette into the *dlg* coding region.

blotting using a radiolabelled 0.5 kb probe which is internal to the *dlg* coding region. The insertion of the 1.5 kb *aphA3* cassette into the *dlg* coding sequence on the GMS800 chromosome is supported by a shift in the *dlg*-containing DNA fragment from 0.5 kb in UA130 to 2.0 kb in the *dlg* knockout mutant (Fig. 4b).

Confirmation of *S. mutans* GMS850, a *fimA*/*dlg* double knockout mutant

Knockout mutations in the *fimA* and *dlg* genes on the *S. mutans* GMS850 chromosome were confirmed by Southern blot analysis. Specifically, *Bam*HI- and *Bgl*II-restricted chromosomal DNAs from UA130 and GMS850 were resolved on a 0.8% agarose gel and transferred to a nitrocellulose membrane which was subsequently hybridized with a 770 bp *fimA*-specific probe. The probe hybridized to a 5.5 kb DNA fragment in the UA130 digest and to a 1.5 kb fragment in GMS850, owing to single *Bam*HI sites present in the *ermAM* and *aphA3* cassettes. This hybridization pattern confirmed the disruption of the *fimA* and *dlg* genes on the GMS850 chromosome (data not shown).

Spot blot analysis

Total RNA isolated from *S. mutans* UA130 and GMS800 grown in THB was spotted onto a nitrocellulose membrane and hybridized with a *fimA*-specific probe. The data revealed increased *fimA* expression in the *dlg*

Fig. 5. Expression of *fimA* is increased in *S. mutans* GMS800 on RNA spot blots. Total RNA was isolated from *S. mutans* UA130 and GMS800 as described and spot blotted onto a nitrocellulose membrane for hybridization with a *fimA*-specific or an *rpsL*-specific probe. The *rpsL* gene, which encodes a constitutively expressed ribosomal protein (Federle *et al.*, 1999), was used as an internal control to normalize comparisons of *fimA* expression across samples. Expression of *fimA* was quantified by scintillation counting and is represented as a proportion of the expression of *rpsL*. Expression studies were performed in duplicate. The results of a single representative experiment are shown.

mutant relative to the UA130 progenitor during the mid-exponential, late-exponential and stationary phases of growth (Fig. 5).

Fig. 6. Expression of FimA is increased in *S. mutans* GMS800 on Western blots. Whole-cell protein isolated from *S. mutans* UA130, GMS700 and GMS800 grown in THB was resolved on a 12.5% SDS-PAGE gel (50 µg per lane), transferred to nitrocellulose, and reacted with a polyclonal antiserum directed against the FimA protein from *S. parasanguis*. The resulting Western blot confirms the absence of the 34 kDa FimA protein from the GMS700 *fimA* knockout mutant, and reveals increased expression of FimA in the GMS800 *dlg* mutant relative to the UA130 wild-type strain.

Western blot analysis of *S. mutans fimA* and *dlg* mutants

Whole-cell protein (50 µg per lane) isolated from *S. mutans* UA130, GMS700 and GMS800 grown in THB was resolved on 12.5% SDS-PAGE gels and reacted with a polyclonal antiserum directed against the *S. para-*

sanguis FimA protein as described in Methods. The resulting immunoblot (Fig. 6) confirms the absence of the 34 kDa FimA protein from GMS700 and reveals increased FimA expression in the GMS800 *dlg* mutant relative to wild-type.

Uptake of ^{55}Fe and ^{54}Mn by *S. mutans*

S. mutans UA130, GMS700 and GMS800 were compared for ^{55}Fe and ^{54}Mn transport in metal ion uptake assays. The bacteria were grown overnight in the presence of ^{55}Fe or ^{54}Mn , washed repeatedly in fresh THB, and the radioactivity associated with bacterial cell pellets measured in a scintillation counter and expressed as a ratio of c.p.m. per c.f.u. Interestingly, uptake of ^{55}Fe by the GMS700 *fimA* knockout mutant was not significantly different from that of the UA130 wild-type progenitor (2.7 ± 0.03 c.p.m. per c.f.u. vs 2.3 ± 0.12 c.p.m. per c.f.u., respectively), possibly owing to other active iron transporters in *S. mutans*. However, we noted significantly increased ^{55}Fe uptake in the GMS800 *dlg* knockout mutant relative to wild-type, (6.0 ± 1.0 c.p.m. per c.f.u. vs 2.3 ± 0.12 c.p.m. per c.f.u., respectively; $P < 0.05$) which was compromised in the GMS850 *fimA/dlg* double knockout mutant (4.7 ± 0.60 c.p.m. per c.f.u. vs 6.0 ± 1.0 c.p.m. per c.f.u.). This finding is consistent with a role for FimA in *S. mutans* iron transport. In contrast, ^{54}Mn uptake was not significantly affected by mutations in *fimA* or *dlg* (data not shown), indicating that neither FimA nor Dlg is likely to be involved in the transport of manganese by *S. mutans*.

S. mutans fimA and *dlg* knockout mutations do not significantly affect caries formation *in vivo*

The cariogenic potential of *S. mutans* GMS700 and GMS800 was examined in germ-free rats. The results of these experiments are summarized in Table 4. The mean

Table 4. Cariogenic potential of *S. mutans fimA* (GMS700) and *dlg* (GMS800) knockout mutants in germ-free rats

Rats were provided a diet containing 5% sucrose (diet 305) and killed on experimental day 35 (when 55 d old). Values are means $\pm$ SEM. Mean caries scores are the extent of caries activity on molar surfaces involving enamel (E), dentinal slight (Ds), dentinal moderate (Dm) and dentinal extensive (Dx) lesions. Bacteria were enumerated on Mitis Salivarius (MS) agar plates.

Infecting strain (group)	No. of rats	Weight (g)	10 ⁴ × Plaque bacteria (c.f.u. ml ⁻¹)		Mean caries score											
					Buccal				Sulcal				Proximal			
			MS	MS + Kan	E	Ds	Dm	Dx	E	Ds	Dm	Dx	E	Ds	Dm	Dx
<i>S. mutans</i> UA130 (group A)	7	157 $\pm$ 9	3380.0 $\pm$ 1047.8	1.3 $\pm$ 0.5	17.6 $\pm$ 0.9	15.1 $\pm$ 0.7	11.9 $\pm$ 0.6	9.3 $\pm$ 0.9	20.6 $\pm$ 0.5	17.9 $\pm$ 0.4	12.4 $\pm$ 0.5	7.4 $\pm$ 1.0	4.4 $\pm$ 0.7	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
<i>S. mutans</i> GMS700 (group B)	7	142 $\pm$ 8	2162.9 $\pm$ 406.3	1984.6 $\pm$ 362.9	15.1 $\pm$ 0.6	13.1 $\pm$ 0.6	9.1 $\pm$ 0.6	6.0 $\pm$ 0.7	21.1 $\pm$ 0.3	17.9 $\pm$ 0.3	11.7 $\pm$ 0.3	6.3 $\pm$ 0.4	4.9 $\pm$ 0.7	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
<i>S. mutans</i> GMS800 (group C)	7	140 $\pm$ 10	77.4 $\pm$ 41.4	71.4 $\pm$ 41.1	19.9 $\pm$ 0.9	17.6 $\pm$ 0.9	14.1 $\pm$ 0.9	10.9 $\pm$ 1.5	22.6 $\pm$ 0.7	20.4* $\pm$ 0.4	14.1 $\pm$ 0.6	8.9 $\pm$ 0.9	5.3 $\pm$ 0.7	1.6 $\pm$ 0.6	0.3 $\pm$ 0.3	0.0 $\pm$ 0.0

* Caries score significantly different ($P \leq 0.05$) between groups A and C.

caries scores for rats infected with either knockout mutant were not significantly different from those infected with the wild-type UA130 progenitor.

DISCUSSION

With the known exceptions of *Lactobacillus plantarum* (Archibald, 1983) and *Borrelia burgdorferi* (Posey & Gherardini, 2000), all living organisms have an absolute requirement for iron (Weinberg, 1978). The concentration of free iron in the human host is only 10^{-12} μ M, however, resulting in conditions of iron starvation for invading microbes. Micro-organisms must therefore respond to iron limitation in the host environment for survival, either by secreting small, iron-chelating molecules (siderophores), or by robbing iron directly from host iron-binding proteins. The iron-withholding system of the human host is also an important environmental signal to which many bacteria respond by expressing a variety of virulence-associated genes (Litwin & Calderwood, 1993).

Previous work in our laboratory applied inductively coupled argon plasma (ICAP) analysis (Coish & Sinton, 1992) to reveal the iron content of human saliva. We defined salivary iron concentrations as ranging from 0.1 to 1.0 μ M (unpublished observations), and therefore included these concentrations in our *in vitro* studies. The growth of *S. mutans* was restricted in a medium containing 0.4 μ M iron, indicating that *S. mutans* is likely to experience iron starvation in the oral cavity, especially during non-mealtimes. The results of dialysis bag and chrome azural assays performed in our laboratory support a receptor-mediated mechanism for *S. mutans* iron uptake/transport (unpublished observations), which is consistent with previous reports in the literature indicating that *S. mutans* does not elaborate siderophores (Evans *et al.*, 1986). Moreover, ferric chloride, ferric/ferrous citrate and transferrin all proved to be suitable iron sources for *S. mutans* growth in iron-starved cultures (data not shown). This is supported by reports that describe a membrane-associated flavin reductase in *S. mutans* that reduces ferric (III) iron into its more soluble ferrous (II) form prior to internalization (Evans *et al.*, 1986). A transferrin receptor has not yet been identified in *S. mutans*, however, nor has one been described in other Gram-positive pathogens known to utilize transferrin as an iron source (Williams & Griffiths, 1992).

The FimA lipoprotein is encoded by the last of three genes which comprise a tricistronic ABC transporter operon on the *S. mutans* chromosome (Kitten *et al.*, 2000). The first ORF shares up to 62% amino acid identity with other streptococcal ATP-binding proteins, including ORF1 in *S. gordonii* (Kolenbrander *et al.*, 1994) and PsaB in *S. pneumoniae* (Novak *et al.*, 1998). The second shares up to 81% amino acid identity with other streptococcal hydrophobic transmembrane proteins within ABC transporter operons, including PsaA in *S. gordonii* (Kolenbrander *et al.*, 1994) and FimB in *S.*

parasanguis (Froeliger & Fives-Taylor, 2000). The *S. mutans fimA* gene product shares up to 76% amino acid identity with other streptococcal LraI proteins, including ScbA in *S. cristae* (Correia *et al.*, 1996), Adc in *S. pneumoniae* (Dintilhac & Claverys, 1997), ScaA in *S. gordonii* (Kolenbrander *et al.*, 1994), and FimA in *S. parasanguis* (Fenno *et al.*, 1995), all of which encode high-affinity metal ion transport proteins. That a *fimA* null mutation was not lethal in *S. mutans* indicates that other mechanisms for iron uptake/transport are functional in this oral pathogen. The presence of other iron transporters in *S. mutans* is also supported by the equivalent amounts of ^{55}Fe uptake we observed for the *fimA* knockout mutant and its wild-type progenitor.

The results of Western and Northern blot analyses reveal an inverse relationship between *S. mutans fimA* expression and iron availability. This is consistent with a role for iron in the regulation of *fimA* expression. In fact, located downstream of the *S. mutans fimA* operon is a 654 bp *dtxR*-like gene (*dlg*) which shares up to 54% similarity at the amino acid level with other iron-dependent repressor proteins including SirR in *Staphylococcus epidermidis* (Cockayne *et al.*, 1998), TroR in *Treponema pallidum* (Hardham *et al.*, 1997) and DtxR in *Corynebacterium diphtheriae* (Tao & Murphy, 1994). Upstream of the *dlg* start codon are putative Shine-Dalgarno, and -10 and -35 consensus sequences, indicating that *dlg* expression may be driven by an independent promoter. Within the promoter region that precedes the *S. mutans fimA* operon is a 38 bp inverted repeat (IR) sequence to which DtxR-like metalloregulatory proteins typically bind (Kitten *et al.*, 2000). This suggests that expression of *dlg* may also be controlled by the promoter that drives expression of the ABC transporter operon, resulting in transcriptional readthrough of *fimA*. Taken collectively, the structural organization of the *S. mutans fimA* locus is consistent with a role for the *dlg* gene product in regulating iron-responsive *fimA* expression in *S. mutans*.

Gram-negative pathogens typically use Fur, a 17 kDa cytosolic metalloregulatory protein that is also regulated by iron, to modulate the expression of genes important for virulence (Bullen *et al.*, 1978; Finkelstein *et al.*, 1983). Western blots performed in our laboratory revealed no evidence of a Fur-like homologue in *S. mutans* but confirmed the presence of a DtxR-like metalloregulator (data not shown). In *Corynebacterium diphtheriae*, DtxR utilizes iron to regulate virulence genes whose products are necessary for adherence and toxin production (Tao & Murphy, 1994). MntR and IdeR are DtxR-like proteins in *Bacillus subtilis* (Que & Helmann, 2000) and *Mycobacterium tuberculosis* (Pohl *et al.*, 1999), respectively, which regulate manganese or iron uptake/transport functions. In the present study, we propose a model for Dlg metalloregulation in *S. mutans* that may involve the formation of Dlg-Fe²⁺ complexes. Specifically, we propose that when iron is plentiful, Dlg-Fe²⁺ complexes form and associate with the IR sequence upstream of the *S. mutans fimA* operon, thereby decreasing iron uptake by down-regulating

Fig. 7. Proposed metalloreulation of the *S. mutans* *fimA* operon by Dlg. (a) In the presence of Fe^{2+} , we propose that Dlg- Fe^{2+} complexes form and bind to an inverted repeat sequence (*dbs1*) located upstream of the *S. mutans* *fimA* operon. This would prevent transcription of the operon by RNA polymerase, and so inhibit expression of *fimA* and possibly other virulence genes within this putative regulon. (b) In the absence of Fe^{2+} , Dlg- Fe^{2+} complexes are not likely to form. As a result, the *S. mutans* *fimA* operon would become derepressed so that *fimA* may be actively transcribed.

expression of the *fimA* iron transporter (Fig. 7a). In contrast, when iron is limiting, as it is in the human host, we propose that Fe^{2+} is not available as a co-repressor to complex with Dlg. Thus, Dlg cannot bind at the IR sequence and *fimA* expression is likely to become derepressed, thereby promoting iron scavenging (Fig. 7b). Evidence supporting this model includes the increase in *fimA* expression noted for the GMS800 *dlg* knockout mutant on Western and Northern blots, and the increase in ^{55}Fe uptake by GMS800 which is compromised in the *fimA/dlg* double knockout mutant, GMS850. Taken collectively, these findings indicate that Dlg is a repressor of *S. mutans* *fimA* expression, and that the *fimA* gene product is involved in iron transport in this oral pathogen. Gel mobility shift assays are currently under way in our laboratory to confirm the *S. mutans* IR sequences as putative Dlg-binding sites.

ABC transporter systems with a requirement for manga-

nese have been described in *S. gordonii* (Kolenbrander *et al.*, 1998), *S. parasanguis* (Fenno *et al.*, 1995) and *S. pneumoniae* (Dintilhac *et al.*, 1997), and conserved nucleotides within the *S. mutans* *dlg* sequence support a putative interaction between Dlg and Mn^{2+} . In addition, a manganese requirement for *S. mutans* is supported by the Mn^{2+} specificity of its superoxide dismutase (Nakayama, 1992). However, while growth-curve experiments in our laboratory support manganese as an essential micronutrient for *S. mutans* growth (unpublished observations), the results of metal ion uptake assays do not implicate the *S. mutans* *fimA* ABC transporter or Dlg in manganese uptake.

Finally, the disruption of *dlg* did not affect *S. mutans*-induced cariogenesis in germ-free rats. This is not surprising since the expression of virulence factors is paramount for bacterial survival in the host environment. Indeed, the expression of genes whose products promote *S. mutans* caries development is likely to be subject to multiple mechanisms of control, not all of which belong to the proposed Dlg regulon. We also noted that the cariogenic potential of *S. mutans* was not significantly affected by a *fimA* knockout mutation in a rat caries model. This is consistent with a recent report by Froeliger & Fives-Taylor (2000) suggesting that FimA is not significantly involved in *S. parasanguis* adhesion to saliva-coated hydroxyapatite. However, recent *in vivo* studies support a role for *S. mutans* FimA in an endocarditis model (Burnette-Curley *et al.*, 1995; Kitten *et al.*, 2000). We propose that fimbrial adhesins, rather than the glucan products of sucrose-dependent glucosyl-transferases, mediate adherence when *S. mutans* translocates into the bloodstream. This is supported by previous work in our laboratory which revealed induction of *fimA* expression upon exposure of *S. parasanguis* cultures to horse or bovine serum (unpublished observations). How iron might be involved in mediating the expression of *fimA* under these host conditions has not yet been determined.

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