Manganese-dependent regulation of the endocarditis-associated virulence factor EfaA of *Enterococcus faecalis*

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There is increasing recognition of the emerging role of manganese regulation and acquisition in some pathogenic bacteria. Expression of the *Enterococcus faecalis* endocarditis-associated virulence factor EfaA is induced by growth in serum. It is demonstrated here that expression of the *efaCBA* operon encoding a putative ABC-type transporter is regulated by Mn²⁺. Transcription of *efaCBA* and EfaA production were repressed in Mn²⁺-supplemented medium. A Mn²⁺-responsive transcriptional regulator, EfaR, sharing 27 % identity with the *Corynebacterium diphtheriae* diphtheria toxin repressor (DtxR), was identified. In the presence of Mn²⁺, EfaR protein bound *in vitro* to the *efaC* promoter region. Analysis of the *E. faecalis* V583 genome revealed ten additional putative EfaR-binding sites, suggesting that manganese availability could have a broader regulatory role in infection. The results identify a new Mn²⁺-sensing regulator in enterococci that regulates the expression of a virulence factor implicated in enterococcal endocarditis.

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INTRODUCTION

The importance of enterococci as pathogens, particularly in the nosocomial setting, is now widely recognized (Murray, 1990) and their increasing antibiotic resistance is narrowing options for treatment of serious infections (Landman & Quale, 1997; Swartz, 1994). The enterococci, especially *Enterococcus faecalis*, are a leading cause of infective endocarditis, accounting for up to 20 % of cases (Mylonakis & Calderwood, 2001). Studies of *E. faecalis* strains revealed a dominant 37 kDa antigen recognized by sera from patients with infective endocarditis (Aitchison *et al.*, 1987). The endocarditis-specific expression of the 37 kDa antigen was confirmed and exploited in a serodiagnostic ELISA, which successfully discriminated cases of *E. faecalis* endocarditis from endocarditis due to other streptococci and from other *E. faecalis* infections such as those in the urinary tract (Shorrock *et al.*, 1990). The gene encoding this antigen has been cloned, sequenced and designated *efaA* (Lowe *et al.*, 1995). Northern blot analysis indicated that its expression was induced by growth of the cells in medium supplemented with human serum (Lowe *et al.*, 1995). Studies with a mouse model of peritoneal infection suggest that EfaA is a virulence factor (Singh *et al.*, 1998). The *efaA* gene has since been found

to be the third gene of the *efaCBA* operon, likely encoding an ABC-type transporter, with EfaA being the lipoprotein component. Recent evidence indicates that some ABC-type transporters homologous to *efaCBA* are high-affinity permeases for divalent transition metal ions. For example, ScaCBA (*Streptococcus gordonii*; Kolenbrander *et al.*, 1998) and MntCAB (*Synechocystis* sp. PCC 6803; Bartsevich & Pakrasi, 1996) have been shown to transport Mn²⁺. In many cases, prokaryotic metal cation homeostasis is achieved by transcriptional regulation of genes encoding metal cation uptake proteins. Two main families of metalloregulators responsive to the divalent transition metal cations Fe²⁺, Mn²⁺ and/or Zn²⁺ have been recognized in Gram-positive bacteria: the Fur and DtxR families. Some members of the DtxR family have been shown to be manganese-dependent transcriptional repressors of ABC metal ion permease operons homologous to *efaCBA*, e.g. ScaR (*Streptococcus gordonii*; Jakubovics *et al.*, 2000) and MntR (*Staphylococcus aureus*; Horsburgh *et al.*, 2002).

In this work, we have tested the hypothesis that *efaA* expression is manganese ion-dependent and regulated via a cation-dependent DtxR-like protein.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Enterococcal cultures were grown at 37 °C without shaking in brain/heart infusion (BHI)

Abbreviations: CBP, calmodulin-binding protein; EMSA, electrophoretic mobility shift assay.

The GenBank/EMBL/DDBJ accession number for the *efaR* gene sequence of *E. faecalis* JH2-2 reported in this paper is AF409093.

Table 1. Strains, plasmids and oligonucleotides used in this studyAbbreviations: Amp^R, beta-lactam resistant; Cam^R, chloramphenicol resistant; Nal^R, nalidixic acid resistant; Tet^R, tetracycline resistant.

Component	Description	Source/reference
Strains		
<i>Enterococcus faecalis</i> JH2-2	Wild-type, standard laboratory strain, plasmid-free	Jacob & Hobbs (1974)
<i>Escherichia coli</i> DH5α	<i>endA1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> (Nal ^R), <i>relA1</i> , $\Delta(lacZYA-argF)$, <i>U169</i> , <i>deoR</i> ⁺	Promega
<i>E. coli</i> XL-1 Blue	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i> , <i>lac</i> [F' <i>proAB</i> , <i>lacI</i> ^q Δ M15 Tn10 (Tet ^R)]	Stratagene
<i>E. coli</i> BL21(DE3)pLysS	F ⁻ , <i>ompT</i> , <i>hsdS_B</i> (r _B ⁻ , m _B ⁻), <i>dcm</i> , <i>gal</i> , λ (DE3), [pLysS Cam ^R]	Stratagene
Plasmids		
pGEM-T	PCR cloning vector, Amp ^R	Promega
pGEM: <i>efaCp</i>	pGEM-derived, contains <i>E. faecalis efaC</i> promoter region and internal region of <i>efaC</i> for use as control	This study
pCal-c	Amp ^R , ColE1 <i>ori</i> , calmodulin-binding peptide (CBP)	Stratagene
pCal-c: <i>efaR</i>	Amp ^R , derived from pCal-c, contains <i>efaR</i> ORF with CBP fused to 3' end of ORF	This study
pSK+: GP19	Derived from pBluescript SK(+), contains <i>efaA</i>	Lowe <i>et al.</i> (1995)
Oligonucleotides*		
YLL11F	5'-Cg <u>ggg</u> ATccGTTTTCCTCACTTTCTCTAATCAACGG	<i>efaR</i>
YLL11R	5'-CgGgCtagcAATCGCGAAGACTATTTAAATTAATT	<i>efaR</i>
YLL14F	5'-GgGgaAttCAAGCGCTTGTATTTAGGTGC	<i>efaC</i> promoter
YLL14R	5'-GgGgaaTTCTTTCTTTCCTGGGCGTTTTA	<i>efaC</i> promoter
NSJ1F	5'-AGCTGTTCAAGCGTTAACCG	<i>efaC</i> internal fragment
NSJ1R	5'-AATCACCTCAGCACCTTGGG	<i>efaC</i> internal fragment

*Bases in lower case indicate mismatched bases introduced into the oligonucleotides to create restriction enzyme sites (underlined).

broth (Oxoid) or tryptone/yeast extract/HEPES-buffered glucose (TYHG) medium [0.25 % (w/v) Bacto-tryptone (Difco), 0.25 % (w/v) yeast extract, 0.5 % (w/v) glucose and 25 mM HEPES-NaOH, pH 7.3]. Metal ion depletion was achieved by adding Chelex-100 resin (Bio-Rad) to 80 g l⁻¹ and stirring overnight at 4 °C. The medium was supplemented with 1 mM MgCl₂ and MnCl₂, CuCl₂, FeCl₃, NiCl₂ and ZnCl₂ were added to 10 µM as appropriate. Glassware was treated by soaking overnight in 0.01 % (w/v) EDTA, followed by six rinses with MilliQ water.

DNA and RNA manipulations. Enterococcal chromosomal DNA was extracted from cells using the method of Skjold *et al.* (1987). Other procedures were performed according to the methods described by Sambrook *et al.* (1989). For Northern blot analysis, primers NSJ1F and NSJ1R (Table 1) were used to produce a 483 bp internal fragment of *efaC*. RNA extraction and Northern blotting were performed as described previously (Jakubovics *et al.*, 2000). The entire *efaR* coding region (672 bp; accession no. AF409093) was amplified using primers YLL11F and YLL11R (Table 1), ligated into pGEM-T (Promega) to produce pGEM: *efaR* and transformed into *Escherichia coli* DH5α. To produce recombinant EfaR protein, pGEM: *efaR* was digested and ligated into *NheI*- and *Bam*HI-digested pCal-c (Stratagene), generating pCal-c: *efaR*. This was transformed into *E. coli* BL21(DE3)pLysS and transformants were selected on LB agar containing ampicillin (50 µg ml⁻¹). For gel-shift analysis, primers YLL14F and YLL14R (Table 1) were used. These span the *efaC* start site, identified by BLAST searching the unfinished and non-annotated sequence of the *E. faecalis* V583 chromosome at the TIGR website (<http://www.tigr.org>). The amplicon was cleaved with *HincII* to yield a 131 bp target incorporating the 97 bp

upstream of the *E. faecalis efaC* start codon and a 212 bp internal fragment for use as a control.

Sequencing and sequence analysis. Sequencing of plasmids constructed in this study was carried out at the automated DNA sequencing facility in the Department of Biology and Biochemistry at the University of Bath. Sequence analyses were performed using the Wisconsin Genetics Computer Group (GCG) software package. EfaB, EfaC and the *E. faecalis* DtxR-like protein EfaR were identified by BLAST search as described above. The BLAST search facilities of the National Library of Medicine, Washington, DC, USA (NCBI) were used to search for homologues.

Protein isolation and Western blotting. To extract surface proteins, enterococcal cells harvested in late exponential phase were digested in TE buffer with lysozyme (0.2 mg ml⁻¹) for 10 min at 37 °C and then vortexed with 0.2 g glass beads ($\leq 10^6$ µm; Sigma). After the beads had settled, the suspension was collected and unbroken cells were pelleted by centrifugation for 1 min at 8000 g. The supernatant was recovered and centrifuged at 13 000 g for 30 min at 4 °C to pellet the envelope fragments. Western blots of the envelope fragments separated by SDS-PAGE on 10 % (w/v) polyacrylamide gels (10 µg protein per lane) were probed with a 1:50 dilution of monospecific polyclonal rabbit antibodies to EfaA. The antiserum was raised in New Zealand White rabbits against EfaA protein. Rabbits were immunized with 100 mg protein on days 1, 7, 14 and 21 with alum as an adjuvant. Serum was collected on day 28. The protein had been expressed from pSK+:GP19 in *E. coli* XL-1 Blue (Lowe *et al.*, 1995), separated by SDS-PAGE and electroeluted from the gel. The blots were visualized using anti-rabbit IgG conjugated

to horseradish peroxidase and enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Life Science).

Purification of recombinant EfaR. LB broth was inoculated 1:50 from an overnight culture of *E. coli* BL21(DE3)pLysS cells harbouring pCal-c: *efaR* and shaken at 200 r.p.m., 37 °C to an OD₆₀₀ of 0.5–0.6. IPTG was then added to 1 mM and the cultures incubated for a further 3–5 h. The cells were pelleted and resuspended in 15 ml CaCl₂ binding buffer (150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, 0.1 mM PMSF, 50 mM Tris/HCl, pH 8.0). Lysozyme (0.2 mg ml⁻¹), RNase A (10 µg ml⁻¹) and DNase I (5 µg ml⁻¹) were added and the suspension was incubated with shaking at 4 °C for 15 min, followed by sonication. Insoluble material was removed by centrifugation. Calmodulin-affinity resin (Stratagene) was used to purify the recombinant EfaR–calmodulin-binding peptide (EfaR–CBP) from the supernatant in accordance with the manufacturer's instructions. Following affinity-purification, the CBP tag was cleaved using thrombin and removed.

Electrophoretic mobility shift assay (EMSA). Target DNA fragments were labelled with [α -³²P]dATP (6000 Ci mmol⁻¹) using the Klenow fragment of DNA polymerase and EMSAs were performed as described previously (Jakubovics *et al.*, 2000).

RESULTS

Nucleotide and amino acid sequence analysis of *efaCBA*

The entire nucleotide and amino acid sequences of the *efaCBA* operon have been compiled from the *E. faecalis* V583 chromosome at the TIGR website (<http://www.tigr.org>). The organization of the *efa* operon is depicted in Fig. 1(a). The *efaA* gene is the third of three genes, which sequence homology suggests encode an ABC-type metal ion transport system. The first gene, *efaC*, encodes an ATP-binding protein (ATPase); the second, *efaB*, encodes a hydrophobic transmembrane protein. EfaA probably functions as a solute binding-protein receptor for the ABC complex. Within the region upstream of *efaC* are putative Shine–Dalgarno, –10 and –35 consensus sequences and two interrupted palindromic sequences of dyad symmetry that we have termed EfaR boxes (Fig. 1b).

EfaC possesses characteristics expected of ATP-binding proteins: a Walker A motif (GPNGAGKST; consensus is GXXGXGKST), the ABC family signature sequence (LSGG, identical to consensus), a Walker B motif (VIFLDEPF; consensus is hhhhDEPT, where h is any hydrophobic amino acid) and a 'switch' region (VGI; consensus is xGh). CLUSTAL W analysis revealed that EfaB and EfaC are highly homologous to their Psa (*Streptococcus pneumoniae*; respectively 57 and 47 % identical), Fim (*Streptococcus parasanguis*, 54 and 53 %) and Sca (55 and 51 % identical) counterparts.

EfaA expression and *efaCBA* transcription is manganese-regulated

When *E. faecalis* JH2-2 cultures were grown in Chelex-treated BHI supplemented with 10 µM Mn²⁺, stationary phase growth yields (OD₆₀₀ 0.94 ± 0.02) were increased by about 50 % compared with unsupplemented medium

Fig. 1. (a) Schematic of the *efaCBA* locus in *E. faecalis* (not to scale). The *efaB* and *efaA* ORFs overlap by 14 bp. The transcript initiated from the *efaC* promoter terminates downstream of *efaA* (25 kb). The divergently transcribed *prs* gene encodes a putative 5-phospho-D-ribose-1-pyrophosphate (PRPP) synthetase. (b) Nucleotide sequence of the *efaCBA* promoter region. Putative EfaR boxes, –35 and –10 elements, ribosome-binding site (RBS) and start codon are indicated.

(OD₆₀₀ 0.637 ± 0.03). Supplementation with other metal cations tested (Fe³⁺, Cu²⁺, Co²⁺, Ni²⁺ or Zn²⁺) at 10 µM had no such effect (OD₆₀₀ 0.615 ± 0.01).

Western blot analyses of cell surface protein extracts from *E. faecalis* JH2-2 grown in Chelex-treated BHI supplemented with metal cations demonstrated that EfaA was strongly expressed in the absence of Mn²⁺. Addition of 10 µM Mn²⁺ to the growth medium repressed its expression (Fig. 2a). In contrast, addition of 10 µM Fe³⁺, Co²⁺, Cu²⁺, Ni²⁺ or Zn²⁺ did not affect EfaA production.

Northern analysis of total RNA from *E. faecalis* JH2-2 using an *efaC* probe revealed *efaCBA* to be transcribed as a single polycistronic transcript of approximately 2.5 kb. In agreement with the Western blot data, the *efaCBA* transcript was strongly expressed when *E. faecalis* JH2-2 was grown in Chelex-treated broth. This transcript was repressed below detectable levels in 10 µM Mn²⁺-supplemented medium (Fig. 2b). In contrast, supplementation with Fe³⁺, Cu²⁺, Ni²⁺ or Zn²⁺ had no apparent effect on the level of transcript production.

Identification of EfaR and production of recombinant protein

Examination of the region upstream of *E. faecalis* *efaC* revealed two inverted repeat sequences that closely resemble binding sequences for a number of metalloregulatory proteins, including DtxR (*Corynebacterium diphtheriae*; Lee *et al.*, 1997), SirR (*Staphylococcus epidermidis*; Hill *et al.*, 1998) and MntR (*Staphylococcus aureus*; Horsburgh *et al.*, 2002). Hence, it was hypothesized that the manganese-regulation of *efaCBA* expression was mediated via a DtxR-like regulator. A DtxR homologue was identified in the *E. faecalis* V583

Fig. 2. (a) Western blot analysis of EfaA expression. Lanes: 1 and 2, *E. coli* XL-1 Blue pSK+:GP19 before (lane 1) and after (2) IPTG induction; 3–10, *E. faecalis* JH2-2 grown in untreated BHI (3) or in Chelex-treated BHI and EDTA-treated glassware unsupplemented (4) or supplemented with 10 µM Mn²⁺ (5), Fe³⁺ (6), Mn²⁺ and Fe³⁺ (7), Cu²⁺ (8), Ni²⁺ (9) or Zn²⁺ (10). (b) Northern blot analysis of *efaCBA* transcription. Cells were cultured in Chelex-treated TYHG. RNA extracts (10 µg per lane) were probed with an internal fragment of *efaC*. Mn²⁺ (lane 2), Fe³⁺ (3), Ni²⁺ (4), Cu²⁺ (5) or Zn²⁺ (6) was added to 10 µM. Lane 1 contained no added metals.

chromosome at TIGR (<http://www.tigr.org>) and the sequence information used to clone the gene from *E. faecalis* JH2-2. The protein, designated EfaR (for *Efa* Regulator-of-expression), comprises 222 amino acid residues with a predicted molecular mass of 25.5 kDa. The sequence of EfaR is 27 % identical (45 % similarity) to that of *C. diphtheriae* DtxR and 39 % identical (56 % similarity) to *Streptococcus gordonii* ScaR.

The pCal-c vector (Stratagene) was chosen to produce recombinant EfaR. This plasmid enabled expression of the cloned protein as a fusion with a 4 kDa C-terminal CBP tag. The CBP tag binds calmodulin with high affinity in the presence of low concentrations of calcium, facilitating recovery of the chimeric EfaR–CBP product by affinity-purification with calmodulin-affinity resin. The CBP tag contains a recognition site for the site-specific protease thrombin, releasing the recombinant protein without any C-terminal modification. After treatment with thrombin, purified EfaR with an apparent molecular mass of 26 kDa was visualized by SDS-PAGE (Fig. 3).

EfaR binds the *efaC* promoter *in vitro* in a metal-ion-dependent manner

Despite the apparent high overall conservation of domain structure and metal-ion-binding residues across the DtxR family of proteins, the various DtxR homologues appear to have different and specific metal ion preferences *in vivo*, although purified DtxR homologues can bind a range of divalent transition metal cations *in vitro* (Hill *et al.*, 1998; Jakubovics *et al.*, 2000). Hence, a 131 bp DNA binding target, including 97 bp upstream of the *efaC* translational start site and designated *efaCp*, was incubated with 0–1.0 µM purified EfaR and 125 µM Mn²⁺ and a concentration-dependent shift

Fig. 3. SDS-PAGE of EfaR–CBP fusion protein and EfaR following cleavage of the CBP tag. An EfaR–CBP fusion protein was over-expressed in *E. coli* BL21(DE3)pLysS and purified using calmodulin-affinity resin. The CBP tag was cleaved with thrombin and removed by binding it to the affinity resin. EfaR protein was then concentrated using a Vivaspin column containing a filter with a 10 000 Da molecular mass cut-off. The purified proteins were electrophoresed on a denaturing 10 % (w/v) polyacrylamide gel and stained with Coomassie blue. Lanes: M, molecular mass marker (sizes in kDa); 1, purified EfaR–CBP; 2, purified and concentrated EfaR following cleavage of CBP.

was seen (Fig. 4a). Metal ion chelation with 1 mM EDTA (Fig. 4b) or competition with non-labelled *efaCp* (Fig. 4c) resulted in loss of shift. A number of other divalent metal cations were tested (Fig. 5); Ni²⁺, Zn²⁺, Co²⁺ and Cu²⁺ also caused EfaR to retard DNA migration. There was no shift when the EfaR promoter region was tested, indicating that the gene is not autoregulated (data not shown).

DISCUSSION

Divalent transition metal ions such as Mn²⁺, Fe²⁺ and Zn²⁺ are essential for both the growth and survival of micro-organisms. Amongst other things, Mn²⁺ is important as a co-factor for many enzymes (Jakubovics & Jenkinson, 2001) and is emerging as a regulator of oxidative stress regulons, for example in *Staphylococcus aureus* (Horsburgh *et al.*, 2001) and *Streptococcus gordonii* (Jakubovics *et al.*, 2002).

Expression of EfaA has previously been shown to be induced by growth of *E. faecalis* in medium containing serum (Lowe *et al.*, 1995). Analysis of the *E. faecalis* V583 genome shows that EfaA is the third component of a trigenic operon homologous to ATP-binding cassette transporters that, in other micro-organisms, have been shown to transport Mn²⁺, e.g. ScaCBA of *Streptococcus gordonii* (Jakubovics *et al.*, 2000) and MntCAB of *Synechocystis* sp. PCC 6803 (Bartsevich & Pakrasi, 1996). We propose that EfaCBA is a manganese-regulated operon that likely functions as a high-affinity manganese permease in *E. faecalis*, possibly playing a role in the infection of human host tissues, where Mn²⁺ availability can be as low as 20 nM (Krachler *et al.*, 1999). Expression of a Mn²⁺ transporter would be expected to be responsive to Mn²⁺ availability in the growth medium, i.e. Mn²⁺ would regulate *efaCBA* expression. Western and Northern blot analyses are consistent with this hypothesis: the addition of 10 µM Mn²⁺ to the medium suppressed EfaA expression and *efaCBA* transcription. The increase in yield of *E. faecalis* JH2-2 cultures when grown in Mn²⁺-supplemented Chelex-treated BHI also indicated that Mn²⁺ is an important micronutrient for this micro-organism. These

Fig. 4. EMSA of EfaR binding to the upstream region of *efaC* (*efaCp*). (a) Effect of EfaR concentration. Purified EfaR was incubated with α -³²P-labelled *efaCp* and 125 μM Mn²⁺. (b) Effect of EDTA on EfaR binding to *efaCp*. α -³²P-labelled *efaCp* was incubated with 1 μM EfaR, 125 μM Mn²⁺ and 1 mM EDTA. (c) Competition with unlabelled *efaCp*. Purified EfaR (1 μM) was incubated with α -³²P-labelled *efaCp* and 125 μM Mn²⁺. Ten μg unlabelled *efaCp* (lane 3) and 10 μg unlabelled control fragment (lane 4) were included.

Fig. 5. Effect of metal cations on EfaR binding to *efaCp*. Purified EfaR (1 μM) was incubated with α -³²P-labelled *efaCp* and 125 μM metal cation.

results suggest that the previously observed serum-mediated induction was likely due to sequestration of Mn²⁺ from the growth medium by components present in serum. Total manganese levels in BHI medium have been estimated to be approximately 26 μM and equilibrium dialysis measurements suggest that only 5 μM is free (Tseng *et al.*, 2001). Levels of available manganese in serum are typically around 20 nM, as most available manganese is complexed with albumin and transferrin (Krachler *et al.*, 1999).

Expression of several *efaCBA* homologues has been shown to be governed by metal-dependent transcriptional regulators belonging to the DtxR family, e.g. in the presence of Mn²⁺, *Streptococcus gordonii scaCBA* transcription is repressed by the DtxR-like protein ScaR (Jakubovics *et al.*, 2000), while *Staphylococcus aureus mntABC* is similarly repressed by MntR (Horsburgh *et al.*, 2002). The archetype of the family, *C. diphtheriae* DtxR, has been shown to bind to 19 bp palindromic elements with the sequence TTAGGTTAGCC-TAACCTAA (Lee *et al.*, 1997). The DtxR consensus binding sequence box 1 is better conserved in the promoter of the *E. faecalis efaCBA* operon than in the promoters of streptococcal homologues (Jakubovics *et al.*, 2000). In fact, the putative binding sequence EfaR box 1, upstream of *efaCBA*, more closely resembles those present in the staphylococci (Hill *et al.*, 1998; Horsburgh *et al.*, 2002) than the 19 bp *C. diphtheriae* consensus binding sequence in that they lack

the central five bases of the consensus sequence. Interestingly, reassessment of the 19 bp motif indicates that it is the result of a repeated CCTAA motif on a 14 bp palindrome.

The results of our EMSAs support the hypothesis that EfaR regulates expression of *efaCBA*. EfaR bound to DNA fragments containing the promoter region of *efaC* but not to non-specific DNA. The various DtxR homologues appear to be specific for either manganese or iron *in vivo* yet, as in this work, most have been observed to bind a range of metal cations *in vitro* (Hill *et al.*, 1998; Jakubovics *et al.*, 2000).

By searching the *E. faecalis* V583 genome for the 14-bp consensus TTAGGNNNCCTAA derived from the EfaR boxes upstream of *efaC*, we have identified potential EfaR boxes in the promoter regions of several genes (Table 2). For example, EfaR boxes were present upstream of the two genes encoding natural resistance-associated macrophage protein (NRAMP) homologues. A similar search in the *Mycobacterium tuberculosis* database yielded over 40 different genes containing putative 19 bp DtxR-like binding sequences (Gold *et al.*, 2001). However, *M. tuberculosis* has two DtxR-like proteins, compared with one in *E. faecalis*. In addition to regulating metal ion transport, *C. diphtheriae* DtxR also regulates expression of virulence genes, the products of which include adhesins and a toxin (Tao *et al.*, 1994). Hence, like DtxR, EfaR may well have a global regulatory role.

Whilst metal ions such as Mn²⁺, Fe²⁺ and Zn²⁺ are essential for many micro-organisms, they can also be potentially toxic at high concentrations. Hence, careful regulation of intracellular concentrations of such cations is essential. Here, we envisage that, in *E. faecalis*, *EfaCBA* is an ABC-type manganese permease regulated by EfaR, with Mn²⁺ acting as a co-repressor. Accordingly, when Mn²⁺ is abundant, intracellular Mn²⁺ levels rise, resulting in the formation of EfaR–Mn²⁺ complexes that bind the *efaC* promoter, inhibiting transcription and hence reducing Mn²⁺ uptake. When Mn²⁺ is scarce or its availability is restricted, e.g. in human serum, the EfaR apoprotein cannot bind the *efaC* promoter, derepressing *efaCBA* expression and hence increasing Efa permease levels and Mn²⁺ scavenging. Attempts to provide supporting genetic evidence have been frustrated by our

Table 2. *E. faecalis* V583 genes possibly containing EfaR boxes upstream of ORF regions designated by EF loci

Locus	EfaR box sequence	Comment
EF2074	TTAGGTGCGCCTAA	Box 1 of <i>efaCBA1</i>
EF2074	TAAGGCAAACCTAA	Box 2 of <i>efaCBA1</i>
EF2073	TTAGGCGCACCTAA	Box 1 of <i>efaCBA1</i> , 88 bp upstream of divergently transcribed <i>prsA</i> , ribose-phosphate pyrophosphokinase, purine biosynthesis
EF2073	TTAGGTTTGCCTTA	Box 2 of <i>efaCBA1</i> , 143 bp upstream of <i>prsA</i> , ribose-phosphate pyrophosphokinase
EF0575	TTAGGCTTGACTAA	<i>efaCBA2</i> , putative Mn transporter, 69 bp upstream of ORF
EF1901	TTAGGTGTGCCTAA	<i>mntH1</i> , putative Mn transporter, 64 bp upstream of ORF
EF1057	TTAGGTGTACCTAA	<i>mntH2</i> , putative Mn transporter, 73 bp upstream of ORF
EF0115	TTAGGAATGCCTTA	Endoribonuclease L-PSP, RNA degradation, 163 bp upstream of ORF
EF1054	TAAGGCTTTCCTAA	ABC transporter permease protein, substrate nitrate (?), 119 bp upstream of ORF
EF1066	ATTGGATAACCTTA	Hexapeptide repeat-containing acetyltransferase, 119 bp upstream of ORF
EF1826	ATTGGTGTTCCTTA	Alcohol dehydrogenase, energy generation by fermentation, 67 bp upstream of ORF
EF3107	ATTGGCAACCCTTA	ABC oligopeptide transporter permease protein, 90 bp upstream of ORF
EF2447	TGCGTACGCCCTAA	ComEC/Rec2 DNA internalization-related competence protein, 85 bp upstream of ORF
EF2434	TTTGGGATTCCTAA	RpiR family phosphosugar-binding transcriptional regulator, 110 bp upstream of ORF
EF3064	TTAGGAACCTACTAA	<i>pnp</i> , polyribonucleotide nucleotidyltransferase, RNA degradation, 92 bp upstream of ORF

inability to generate mutants either in the *efaCBA* operon or *efaR*, despite extensive attempts using both allelic replacement and insertion duplication strategies.

In summary, little has been reported about enterococcal metal requirements. Here, we demonstrate that expression of the endocarditis-associated virulence factor EfaA is manganese-regulated. We have also identified a metalloprotein that binds to the promoter region of the *efaCBA* operon in a Mn^{2+} -dependent manner and therefore likely mediates the manganese-dependent repression of the *efaCBA* operon *in vivo*. Sequences similar to the DtxR consensus binding sequence are also present upstream of several other enterococcal genes, suggesting that EfaR could represent a new paradigm for metalloregulation in the enterococci. The induction of EfaA expression by serum (Lowe *et al.*, 1995) highlights the fact that serum could impose more than just restricted iron availability, and sequestration of other transition metal cations, such as manganese, is also important.

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