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A Rex Family Transcriptional Repressor Influences H_2O_2 Accumulation by *Enterococcus faecalis*

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Rex factors are bacterial transcription factors thought to respond to the cellular NAD^+/NADH ratio in order to modulate gene expression by differentially binding DNA. To date, Rex factors have been implicated in regulating genes of central metabolism, oxidative stress response, and biofilm formation. The genome of *Enterococcus faecalis*, a low-GC Gram-positive opportunistic pathogen, encodes *EF2638*, a putative Rex factor. To study the role of *E. faecalis* Rex, we purified EF2638 and evaluated its DNA binding activity *in vitro*. EF2638 was able to bind putative promoter segments of several *E. faecalis* genes in an NADH-responsive manner, indicating that it represents an authentic Rex factor. Transcriptome analysis of a Δ*EF2638* mutant revealed that genes likely to be involved in anaerobic metabolism were upregulated during aerobic growth, and the mutant exhibited an altered NAD^+/NADH ratio. The Δ*EF2638* mutant also exhibited a growth defect when grown with aeration on several carbon sources, suggesting an impaired ability to cope with oxidative stress. Inclusion of catalase in the medium alleviated the growth defect. H_2O_2 measurements revealed that the mutant accumulates significantly more H_2O_2 than wild-type *E. faecalis*. In summary, EF2638 represents an authentic Rex factor in *E. faecalis* that influences the production or detoxification of H_2O_2 in addition to its more familiar role as a regulator of anaerobic gene expression.

Ongoing bacterial metabolism and growth require maintenance of the redox balance in the cell. To achieve this balance, bacteria have evolved mechanisms to monitor their redox state and convert redox signals into adaptive regulatory outputs. For example, changes in availability of oxygen or metabolic activity can influence the relative levels of the dinucleotides NAD^+ and NADH in the cell (1), and changes in this ratio are detected by the Rex family of transcription factors that are widespread in the genomes of Gram-positive bacteria. Rex factors have been extensively studied in *Streptomyces coelicolor*, *Thermus aquaticus*, *Thermus thermophilus*, *Bacillus subtilis*, and *Staphylococcus aureus* (2–8). Rex factors respond to the cellular NAD^+/NADH ratio to modulate expression of genes involved in anaerobiosis, fermentative metabolism, biofilm formation, and oxidative stress (2–4, 7, 9, 10). Structural studies of Rex factors have identified dinucleotide binding pockets in the C-terminal Rossmann fold domain of the protein. NADH binding in this region leads to a conformational change in a Rex homodimer and a subsequent displacement of Rex from its recognition sites on DNA, leading to derepression of the downstream genes (2–4).

Enterococcus faecalis, a low-GC Gram-positive bacterium and an opportunistic pathogen, is a facultative anaerobe and a commensal member of the gastrointestinal microbiota in insects and animals, including humans (11, 12). *E. faecalis* is a hardy organism that exhibits substantial resistance to diverse environmental stresses, including antibiotics, bile detergents, and oxidative stress. *E. faecalis* can produce large quantities of reactive oxygen species, such as superoxide and hydrogen peroxide (13, 14). To cope with the stress this imposes, *E. faecalis* expresses numerous gene products responsible for detoxification of reactive oxygen species (15), including an NADH peroxidase (16, 17), a heme-dependent catalase that is functional only when heme is available in the medium (18, 19), a manganese-dependent superoxide dismutase (20), and a glutathione reductase (21), among others. As a facultative anaerobic member of the gastrointestinal (GI) tract, *E. faecalis* must adapt to fluctuations in the availability of oxygen and nutrients so that it can proliferate and compete with other community members. However, the mechanisms used by *E. faecalis* to monitor environmental conditions, such as the level of oxygen available, in order to mount an adaptive response are not well understood.

Although Rex factors are widespread in the genomes of Gram-positive bacteria, most genomes encode only one Rex factor homolog. The genome of *E. faecalis*, however, encodes two putative Rex factors (*EF2638* and *EF2933*) that exhibit the characteristic Rex family bipartite domain architecture consisting of an N-terminal DNA-binding domain coupled to a C-terminal domain bearing a dinucleotide-binding Rossmann fold. The mechanisms by which multiple Rex factors in a single organism coordinate with each other to modulate gene expression in response to the cellular redox state are unknown. To our knowledge, the role of the putative Rex factors in *E. faecalis* has not been studied.

In this work, we demonstrate that EF2638 is an authentic Rex factor capable of interacting with DNA in an NADH-responsive manner. Our data indicate that EF2638 has the ability to regulate genes through its DNA binding activity, that EF2638 contributes to NAD^+/NADH homeostasis, and that elevated levels of H_2O_2 impose oxidative stress on the Δ*EF2638* mutant to account for its growth defect under certain environmental conditions. In addition, our results establish that the two Rex factors encoded in the *E. faecalis* genome are not functionally redundant.

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TABLE 1 Plasmids and strains used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
E. coli		
TOP10	*E. coli* routine cloning host	Invitrogen
Electro10	*E. coli* cloning host for pCJK218-based plasmids	Agilent
BL21/DE3	*E. coli* protein expression host	Lab stock
E. faecalis		
OG1	Wild-type reference strain	22
DV87-4	OG1 Δ*EF2638-2*	This work
DV106	DV87-4 (*EF1116-EF1117*)*2*::*EF2638-3*	This work
DV122-1	OG1 Δ*EF2933-2*	This work
Plasmids		
pET28b	*E. coli* expression vector (Kn^r)	Novagen
pCJK3	pTRKL2 derivative with pJMA61-derived transcriptional terminator	This work
pCJK4	Plasmid carrying promoterless *lacZ*	This work
pJRG32	pCJK47 derivative with a synthetic P-*pheS** cassette (Em^r)	23
pVE6007	Temperature-sensitive derivative of pWV01 (Cm^r)	24
pCJK47	*E. faecalis* allelic exchange vector with *pheS** counterselectable marker (Em^r)	25
pCJK218	pLT06 derivative with a synthetic P-*pheS** cassette (Cm^r)	This work
pCJK141	pCJK47 derivative enabling ectopic integration of genes at the *EF1116-7* locus	This work
pDV75-2	pCJK218 derivative enabling ectopic integration of genes at the *EF1116-7* locus	This work
pDV41-1	EF2638 cloned in pET28b	This work
pDV42-4	Δ*EF2638-2* (ΔP7-L211) in pJRG32	This work
pDV92	Δ*EF2933-2* (ΔP6-N211) in pCJK218	This work
pDV59-3	pCJK4::*EF2638p* (424 bp upstream of start codon)-*lacZ*	This work
pCJK221	pCJK4::*EF1929p* (399 bp upstream of start codon)-*lacZ*	This work
pDV80	pDV75-2::*EF2638-3* with C-terminal Strep-tag	This work

MATERIALS AND METHODS

Bacterial strains, growth media, oligonucleotides, and chemicals. The strains and plasmids used in this study are listed in Table 1. Oligonucleotides used for plasmid construction were synthesized by Integrated DNA Technologies, Inc. *Escherichia coli* strains were grown either in LB (Difco) or brain heart infusion (BHI) (Difco) at 37°C with shaking at 225 rpm. *E. faecalis* strains were cultured in Mueller-Hinton (MH) broth prepared according to the manufacturer's instructions (Difco), or MM9YE, a previously described semidefined medium (25) supplemented with 0.3% glucose or 0.3% glycerol (ACROS). Where indicated, MM9YE-based medium was supplemented with bovine catalase (~500 U/ml) or porcine hematin (8 μM). When required, antibiotics were added at the following concentrations: for *E. coli*, 50 μg/ml kanamycin (Kn), 100 μg/ml erythromycin (Em); for *E. faecalis*, 10 μg/ml erythromycin, 10 μg/ml chloramphenicol (Cm). All chemicals and antibiotics were purchased from Sigma unless otherwise indicated.

Plasmid construction. A plasmid (pDV41-1) to overexpress C-terminally tagged EF2638-His_6 for purification from *E. coli* was constructed by amplifying full-length *EF2638* from *E. faecalis* OG1RF genomic DNA and introducing it into pET28b with primer-encoded NcoI/XhoI restriction sites.

To improve upon and extend previously described versions of the markerless allelic exchange technology used for genetic manipulation of *E. faecalis*, modified plasmids for markerless exchange were developed (see Fig. S1 in the supplemental material). First, we exchanged the *pheS** counterselectable marker found in the temperature-sensitive allelic exchange vector pLT06 (26) with a previously described synthetic *pheS** allele (23) bearing synonymous substitutions at the wobble position in many of the codons, to prevent recombination of the plasmid-borne *pheS** with the chromosomal allele, thereby creating pCJK218. Second, we developed a system to stably introduce cloned genes into the chromosome of *E. faecalis* at an ectopic locus. Such a system enables cloned genes to be present in a single copy, and without the burden of maintaining an independent plasmid, for the purposes of complementation analysis. Our system is analogous to a previously described strategy (27), although in our case integration into the *E. faecalis* chromosome occurs at a distinct site from that utilized in the previous report. The system we developed enables genes to be integrated in the intergenic region between the convergently transcribed genes *EF1116* and *EF1117*. This intergenic region encodes a stem-loop transcriptional terminator (28), and in our constructs this terminator is duplicated such that a copy of the terminator flanks both sides of the ectopically integrated sequence to prevent any transcriptional readthrough from (or out into) adjacent genes. The plasmid enabling integration at the *EF1116-7* locus (pDV75-2) was developed in stages: first, segments of the *E. faecalis* OG1RF chromosome to serve as the substrates directing homologous recombination were amplified by PCR. The segments that encode portions of the *EF1116* and *EF1117* genes (~820 bp and ~760 bp, respectively) were assembled together by restriction digest and PCR into an intact fragment in which the two segments are separated by a short multiple-cloning site (the NotI-NcoI fragment from pCJK47). This intact fragment was then introduced into pCJK47 using primer-encoded XbaI/SphI restriction sites, creating pCJK141. Subsequently, the XbaI/SphI fragment from pCJK141 was transferred to pCJK218 by restriction digest of pCJK141 with XbaI to open the plasmid, T4 polymerase treatment to create a blunt end from the XbaI sticky end, and finally an SphI restriction digest to release the now blunt-end/SphI fragment of interest. This fragment was subsequently ligated into SmaI/SphI-digested pCJK218, creating pDV75-2. A derivative of pDV75-2 containing an epitope-tagged version of *EF2638* and its putative promoter (pDV80) was created by first amplifying full-length *EF2638*, including a 424-bp region (containing the promoter) upstream of the start site, and then introducing the fragment into PstI/EcoRI-digested pDV75-2 using primer-encoded PstI/MfeI sites.

A plasmid to construct operon fusions with *lacZ* (pCJK4) was developed by first amplifying a short segment encoding a transcriptional terminator from pJMA61 and introducing it into pTRKL2 (29) using primer-encoded EcoRV/BglII restriction sites, creating pCJK3. Subsequently, a promoterless *lacZ* gene derived from pTRK390 (30) was amplified and introduced into pCJK3 using primer-encoded SmaI/StuI restriction sites. The final plasmid therefore encodes a transcriptional terminator to prevent readthrough into *lacZ* and 3 unique restriction sites (PstI, BamHI, XmaI) to enable cloned promoters to be introduced upstream of *lacZ*. Plasmids pDV59-3 and pCJK221 were constructed by amplifying putative promoter segments for *EF2638* (424 bp upstream of the *EF2638* start codon) and *EF1929* (399 bp upstream of the *EF1929* start codon) and introducing them into pCJK4 using primer-encoded PstI/BamHI (*EF2638*) or PstI/SmaI (*EF1929*) restriction sites.

Construction of the Δ*EF2638* mutant. Modification of the *EF2638* locus in the *E. faecalis* OG1 chromosome was performed using markerless exchange as described previously (25). A derivative of pJRG32 carrying an in-frame deletion allele of *EF2638* (pDV42-4) was constructed using a BsaI-based cloning scheme to seamlessly fuse two PCR amplicons flanking *EF2638* to generate the in-frame deletion. The deletion allele was de-

signed such that the first and last 6 codons remained (94% of the open reading frame [ORF] was deleted). This deletion allele was transferred to the native *EF2638* locus in the OG1 chromosome using pVE6007 as a helper plasmid as previously described (31). Deletion mutants were isolated by plating on counterselection medium containing *p*-Cl-Phe (25) at 30°C, for 2 to 3 days. Two such Δ*EF2638* mutants isolated completely independently from each other were analyzed and found to exhibit identical phenotypes.

Complementation of the Δ*EF2638* mutant. For complementation analysis, a fragment encoding an epitope-tagged *EF2638* and its putative promoter (same promoter used for *lacZ* fusion) was inserted in a single copy into an ectopic locus in the *E. faecalis* chromosome. This fragment was amplified from *E. faecalis* OG1RF genomic DNA and introduced into pDV75-2 using primer-encoded PstI/MfeI restriction sites, creating pDV80. The cloned *EF2638* allele encoded a C-terminal Strep tag epitope tag (WSHPQFEK). A procedure similar to that described by Thurlow and coworkers (26) was used to obtain recombinants in which the cloned fragment had been transferred to the chromosome by recombination. Briefly, pDV80 was introduced into the *E. faecalis* Δ*EF2638* mutant (DV87-4) by electroporation, with selection on BHI supplemented with Cm and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (100 μg/ml) at 30°C. Subsequently, transformants were used to inoculate cultures, grown to optical density at 600 nm (OD_{600}) of ~0.2 and shifted to 42°C for ~3 h. Dilutions were spread on BHI supplemented with Cm and X-Gal (100 μg/ml) at 42°C. Blue colonies were screened for the integration event by PCR, and the integrant colonies were used to inoculate cultures in BHI (30°C, 225 rpm, overnight). The mutants with *EF2638*-Strep integrated ectopically in the *E. faecalis* chromosome were isolated by plating on counterselection plates as described for the construction of the Δ*EF2638* mutant above and confirmed by PCR. Two such complemented mutants isolated completely independently from each other were analyzed and found to exhibit identical phenotypes. In addition, we found that ectopic introduction of wild-type EF2638 (lacking the epitope tag) in the identical fashion also complemented the Δ*EF2638* mutation (not shown).

Construction of the Δ*EF2933* mutant. A mutant lacking *EF2933* was constructed using markerless exchange as described above for the Δ*EF2638* complementation strain. A derivative of pCJK218 carrying an in-frame deletion allele of *EF2933* (pDV92) was constructed using a BsaI-based cloning scheme as described above. The deletion allele was designed such that the first and last 5 codons remained (95% of the ORF was deleted). Deletion mutants were isolated by plating on counterselection medium containing *p*-Cl-Phe (25) at 30°C, for 2 to 3 days. Two such Δ*EF2933* mutants isolated completely independently from each other were analyzed and found to exhibit identical phenotypes.

Overexpression and purification of *E. faecalis* EF2638. Overnight cultures of *E. coli* BL21 [DE3] (pDV41-1) were grown in LB supplemented with 50 μg/ml kanamycin at 37°C, diluted 50-fold into 200 ml of the same medium, incubated 3 h at 37°C at 250 rpm, and then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Gold Biotechnology) for 1 h at 30°C. Bacteria were collected by centrifugation (10,816 × *g*, 8 min, 4°C) and cell pellets resuspended in 7 ml binding buffer (50 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8). Cell suspensions were treated with lysozyme (1 mg/ml) in lysozyme buffer (10 mM Tris, 50 mM NaCl, 10 mM EDTA, pH 8) for 20 min at 37°C. Cells were disrupted by sonication, and debris was removed by centrifugation (26,892 × *g*, 15 min, 4°C) followed by filtration of supernatant through a 0.22-μm-pore-size filter. The filtered supernatant was loaded onto an Ni column (Profinity IMAC Ni-charged resin; Bio-Rad) equilibrated with binding buffer. Columns were subsequently washed with 5 column volumes of binding buffer, followed by 5 column volumes of wash buffer (50 mM Tris, 300 mM NaCl, 20 mM imidazole, pH 8) and eluted in elution buffer (50 mM Tris, 300 mM NaCl, 500 mM imidazole, pH 8). Eluted fractions were analyzed by 10% SDS-PAGE, and fractions containing the protein were dialyzed against dialysis buffer (100 mM MOPS [pH 7.5], 0.8 M LiCl, 1 mM EDTA). Dialyzed protein was stored at −80°C.

Electrophoretic mobility shift assay (EMSA) for DNA binding. Putative promoter segments of *EF0255* (EF0255p), *EF2933* (EF2933p), *EF2638* (EF2638p), and *EF1929-27* (EF1929p) were amplified from *E. faecalis* OG1RF genomic DNA. These segments included the upstream region as well as a few nucleotides into the actual reading frame; if the sequence was much larger than 300 bp, the segments were split into two smaller fragments containing a 30- to 50-bp overlap. The segments corresponded to 260 bp for EF0255p (includes both predicted Rex binding sites), 219 bp for EF2933p, 457 bp for EF2638p, and 304 bp for EF1929p. Double-stranded fragments were labeled at the 3′ end with digoxigenin (DIG) via the DIG gel shift kit, 2nd generation (Roche Applied Science), according to the manufacturer's instructions. DIG-labeled probes (~5 fmol) and purified EF2638-His_6 (1 μM) were incubated in binding buffer (0.1 M MOPS [pH 7.5], 0.8 M LiCl, 1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol) supplemented with 1 μg poly(dI-dC) and 1 μg poly L-lysine, for 30 min at room temperature (RT). The effect of NAD^+, NADH, and NADPH on electrophoretic mobility was tested by the addition of the dinucleotides (10 mM final concentration) to the reaction following an initial incubation in the absence of dinucleotides (15 min, RT). Reactions were then incubated an additional 15 min, RT. Loading dye was added and samples were subjected to electrophoresis at 50 V for 3 h at 4°C on a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA that had been prerun for 15 min at 120 V. After electrophoresis, DNA was transferred onto positively charged nylon membranes (Roche), subjected to UV cross-linking, probed using anti-digoxigenin-alkaline phosphatase-conjugated antibody (Roche), and detected with 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD) (Roche) by following the manufacturer's directions.

β-Galactosidase activity measurements. β-Galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. Overnight cultures of plasmid-bearing strains were diluted to an OD_{600} of 0.01 in MH broth supplemented with erythromycin (10 μg/ml) and cultured until an OD_{600} of 0.2 at 37°C and 225 rpm. Cultures were then chilled on ice and pelleted (1,503 × *g*, 10 min, 4°C). Cells were resuspended in Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $Na_2HPO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-ME). A portion (200 μl) of each sample was treated with 25 μl 0.1% sodium dodecyl sulfate (SDS) and 50 μl chloroform for 10 min at RT. ONPG (200 μl, 4 mg/ml) was added to samples and incubated for 10 min at RT, and reactions were stopped by the addition of 500 μl 1 M Na_2CO_3. Cellular debris was removed by centrifugation (21,130 × *g*, 5 min, RT), and absorbance was measured at 420 and 550 nm; samples were normalized for OD_{600}. Samples were analyzed in triplicate, and experiments were conducted at least two times. Statistical analysis was performed using a two-tailed Student *t* test.

NAD^+/NADH extraction and cycling assay. Dinucleotides were extracted and assayed according to a previously described method (32), with modifications. Cultures growing exponentially were collected by centrifugation (10,816 × *g*, 8 min, 4°C). Cell pellets were washed once with water, collected by centrifugation (16,100 × *g*, 2 min, 4°C), resuspended in 1,100 μl water, and then split into two 500-μl samples and centrifuged again (16,100 × *g*, 2 min, 4°C). Cell pellets were frozen in a dry ice-ethanol bath. NAD^+ and NADH were extracted by the addition of 100 μl 0.2 M HCl and 100 μl 0.2 M NaOH, respectively. Samples were boiled for 10 min and neutralized, and cell debris was collected by centrifugation at 5,000 × *g* for 5 min at 4°C. Control experiments using pure NAD^+ or NADH that had been subjected to acid or base treatment established the specificity of the extraction procedure for the respective nucleotide. Supernatants were transferred to new tubes and kept on ice. Extracts were used immediately in the cycling assay. The reaction mixture for the cycling assay was comprised of 20 μl 1 M bicine (pH 8), 20 μl 16.6 mM PES, 20 μl 4.2 mM MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], 20 μl 100% EtOH, 8 μl H_2O, and 2 μl yeast alcohol dehydrogenase

(yADH). A total of 40 μl of each nucleotide extract was added to the wells of a 96-well plate, samples were treated with 80 μl of the reaction mixture, and absorbance was measured kinetically at 570 nm for 15 min. The concentrations of NAD^+ and NADH were determined using an NAD^+ standard curve, and results are expressed as the NAD^+/NADH ratio. The experiment was done a minimum of two times, and the results represent the means ± standard errors. Statistical analysis was performed using a two-tailed Student *t* test.

Quantitation of hydrogen peroxide. H_2O_2 present in culture supernatants was quantified using Amplex Red (Invitrogen), according to the manufacturer's instructions. Overnight cultures were grown under static conditions in MM9YE supplemented with 0.3% glucose, diluted to an OD_{600} of 0.01 in 75 ml of the same medium in 250-ml flasks. Cultures were incubated either statically or with aeration at 37°C and collected at an OD_{600} of 0.4. After the bacteria were collected by centrifugation (10,826 × *g*, 8 min, 4°C), culture supernatants were filtered through 0.22-μm-pore-size filters prior to analysis with Amplex Red. Results are expressed as H_2O_2 concentration determined using a standard curve generated as per the manufacturer's directions. Standards were prepared in equivalent culture medium. Control experiments in which aliquots of the culture supernatants were treated with bovine catalase (~500 U/ml, 30 min, RT) before Amplex Red analysis established that the Amplex Red signal was indeed due to H_2O_2. The experiments were performed a minimum of two times. Data are presented as means ± standard errors. Statistical analysis was performed using a two-tailed Student *t* test.

Microarray analysis of gene expression. Custom 12- by 135K microarrays (NimbleGen) were designed to interrogate expression of each ORF encoded in the *E. faecalis* OG1RF genome sequence. Although an annotated version of the complete OG1RF genome is now available (NCBI accession no. CP002621), at the time of array design, this annotation was not complete. Consequently, the arrays were designed on the basis of an OG1RF annotation generated with the Rapid Annotation Using Subsystem Technology (RAST) server (33), which has been previously described (34). This annotation contains 2,562 ORFs, of which unique probe sets were successfully designed for 2,477 ORFs. The probe set for most ORFs contains 18 probes per ORF (43,638 probes for all ORFs), each of which is present in triplicate on the array for a total of 130,914 probes per array.

For microarray analysis of gene expression, *E. faecalis* cultures were grown in MH broth at 37°C with aeration to exponential phase (OD_{600} = 0.16). Cultures were rapidly chilled in an ice water bath, and bacteria were subsequently collected by centrifugation. Cell pellets were immediately resuspended in RNAProtect reagent (Qiagen) to kill cells and stabilize the RNA. RNA was recovered from cells using the RNeasy minikit (Qiagen) according to the manufacturer's instructions, with slight modifications. The frozen cell pellets were thawed and resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, pH 8) containing 250 U/ml mutanolysin and 15 mg/ml lysozyme with incubation at 37°C for 10 min. Buffer RLT (Qiagen RNeasy minikit) was then added, and the samples were processed according to the manufacturer's instructions. Purified RNA was subjected to cDNA synthesis, labeling, hybridization to the microarrays, scanning, and normalization via RMA at the Carver Center for Genomics at the University of Iowa. Intensity data from biological replicate cultures, prepared ~1 year apart, were averaged, and genes for which the $\log_2$ of the expression was greater than 2-fold different in the Δ*EF2638* mutant compared to the wild type are included in Table 2. Statistical significance of the differences was assessed via *t* test, and in all cases the *P* value was <0.001.

Microarray data accession number. The microarray data reported here have been deposited in the Gene Expression Omnibus database, under the accession number GSE43228.

TABLE 2 Genes upregulated in the Δ*EF2638* mutant

ORF	RAST annotation	$\log_2$ of ratio[a]
EF0094	Formate/nitrite transporter family protein	2.75
EF0255	L-Lactate dehydrogenase (EC 1.1.1.27)	1.89
EF0475	Ferrous iron transport protein A	2.25
EF0634	Decarboxylase, putative	2.75
EF0635	Amino acid permease family protein	2.83
EF0900	Alcohol dehydrogenase (EC 1.1.1.1); acetaldehyde dehydrogenase (EC 1.2.1.10)	2.11
EF1225	Hypothetical similar to thiamine biosynthesis lipoprotein ApbE	4.73
EF1226	Fumarate reductase, flavoprotein subunit precursor (EC 1.3.99.1)	5.18
EF1227	Fumarate reductase, flavoprotein subunit precursor (EC 1.3.99.1)	3.51
EF1326	Transcriptional regulator, TetR family	2.28
EF1327	BadF/BadG/BcrA/BcrD ATPase family protein	4.89
EF1491	Ribonucleotide reduction protein NrdI	2.26
EF1612	Pyruvate formate-lyase-activating enzyme (EC 1.97.1.4)	3.87
EF1613	Pyruvate formate-lyase (EC 2.3.1.54)	2.36
EF1825	Conserved domain protein	3.21
EF1826	Alcohol dehydrogenase (EC 1.1.1.1)	2.63
EF2048	rRNA large subunit methyltransferase N (EC 2.1.1.-)	2.33
EF2637	Abortive infection protein	3.06
EF2754	Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2)	2.22
EF2755	Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4)	2.16
EF2933	Redox-sensitive transcriptional regulator (AT-rich DNA-binding protein)	2.46
EF3245	Cell envelope-associated acid phosphatase	2.77
EF3198[b]	Lipoprotein, YaeC family	−2.07

[a] The value is $\log_2$ of the ratio (Δ*EF2638* mutant/wild type) of averaged expression values for the two strains.
[b] The single gene with a 2-fold-lower expression in the absence of EF2638.

RESULTS

Transcriptome analysis reveals a role for EF2638 in regulation of fermentative metabolism. To explore the physiological role of the putative *E. faecalis* Rex factor, EF2638, we constructed an in-frame deletion mutant lacking EF2638 in an otherwise wild-type *E. faecalis* strain (OG1). Because Rex factors are known to be transcriptional repressors in other bacterial species, we anticipated that genes normally repressed by EF2638 would exhibit elevated expression in the mutant. Microarray analysis was performed to compare the global transcriptome of the Δ*EF2638* mutant with that of the corresponding wild type during exponential growth in MH broth. Indeed, we found 21 genes encoded at 14 distinct loci whose levels of expression were enhanced by a factor of at least 2 in the absence of EF2638 (Table 2). Strikingly, most of the derepressed gene products are predicted to function primarily in anaerobic fermentative metabolism, including pyruvate formate-lyase (EF1613) and its activating enzyme (EF1612), alcohol/aldehyde dehydrogenase (EF0900), anaerobic ribonucleotide reductase (EF2754-5), subunits of a putative fumarate reductase (EF1226-7), another putative alcohol dehydrogenase (EF1826), and a gene predicted to be involved in anaerobic degradation of glutamate (EF1327). Rex-mediated control of such genes is consistent with observations made previously in other species of bacteria (2, 35, 36) and recent predictions based on bioinformatics analyses (10). We also note that the major lactate dehydrogenase in *E. faecalis* (EF0255) was upregulated in the Δ*EF2638* mutant but did not quite achieve the 2-fold cutoff (1.89-fold). Thus, EF2638 either directly or indirectly controls the expression of nu-

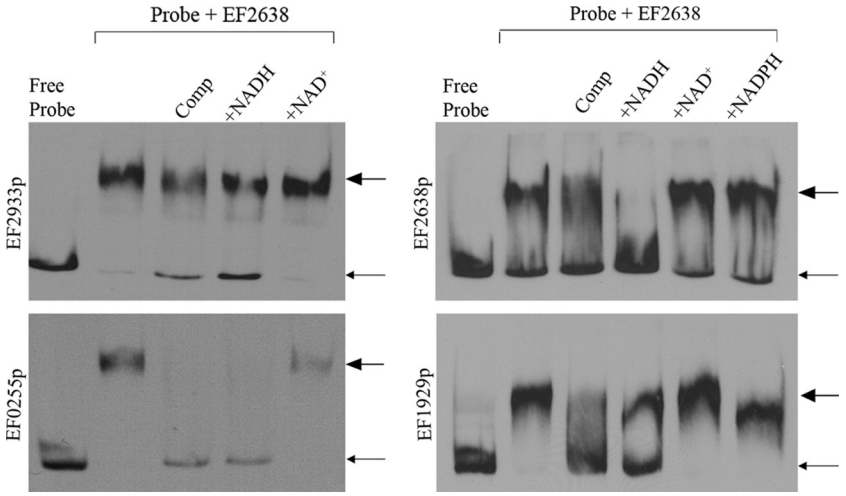

FIG 1 EF2638 binds DNA in an NADH-responsive manner. EMSAs were performed using 3′ DIG-labeled probes containing putative promoter regions of *EF2638* (EF2638p), *EF1929-27* (EF1929p), *EF2933* (EF2933p), or *EF0255* (EF0255p). Pure EF2638-His_6 was incubated with probes in the presence or absence of pyridine dinucleotide as indicated. Comp, unlabeled specific competitor for each probe: EF2638p, EF2933p, and EF0255p at 150× excess and EF1929p at 250× excess. Small arrows indicate the position of free probe, and large arrows indicate the position of probe-DNA complexes. Representative results from at least three independent experiments are shown.

merous enzymes that consume NADH in *E. faecalis*, consistent with the predicted role of Rex factors in maintenance of cellular redox homeostasis. We also observed derepression of the second putative Rex factor in *E. faecalis* (EF2933) in the Δ*EF2638* mutant. There was little evidence in our transcriptome analysis for a role of EF2638 as an activator of gene expression, as only a single gene achieved 2-fold-lower expression in the Δ*EF2638* mutant under the conditions of our experiment.

EF2638 binds DNA in an NADH-responsive manner to regulate gene expression. Because EF2638 possesses a putative DNA-binding domain, we tested whether recombinant, purified *E. faecalis* EF2638-His_6 can bind DNA *in vitro* using electrophoretic mobility shift assays (EMSAs). A series of digoxigenin-labeled probes encompassing several putative promoter regions were prepared. These included 2 probes for genes identified as derepressed in the transcriptome analysis (*EF2933*, *EF0255*) and for which putative Rex-binding sequences had been proposed previously (37), a probe for *EF2638* itself (as Rex homologs are predicted or known to be autoregulated) (7, 10), and a probe for the putative promoter of the *glpKOF* operon (*EF1929-27*), as subsequent growth studies with the Δ*EF2638* mutant (see below) suggested that these genes might be regulated by EF2638 as well. Purified EF2638-His_6 was capable of binding all 4 of the putative promoter segments tested, as indicated by the appearance of a shifted band in the EMSA (Fig. 1), and the addition of excess unlabeled probe competed with the labeled probe for binding, indicating that the interaction was specific. To determine whether EF2638 DNA-binding activity is regulated by the presence of pyridine dinucleotides (as is known to be the case with Rex factors from *S. coelicolor*, *T. aquaticus*, *B. subtilis*, among others) (4, 7, 8), EMSAs were carried out in the presence of NAD^+ and NADH (and NADPH in some cases). Neither NAD^+ nor NADPH exhibited any effect on DNA binding by EF2638, whereas inclusion of NADH led to an obvious decrease in EF2638-DNA complex formation (Fig. 1). Thus, EF2638 exhibits the properties of an authentic Rex family transcriptional repressor, consistent with the hypothesis that EF2638 regulates gene expression in an NADH-responsive manner to maintain redox homeostasis in *E. faecalis*.

The microarray results suggested that EF2638 behaves as a transcriptional repressor for nearly all of the genes exhibiting differential expression. As a further test that EF2638 functions as a repressor *in vivo*, operon fusions of the putative promoters for two of the EMSA-shifted probes (*EF2638* and *glpKOF*) to a promoterless *lacZ* were constructed. The recombinant constructs were introduced into both wild-type and Δ*EF2638* mutant *E. faecalis* strains, and β-galactosidase activity assays were performed, revealing enhanced β-galactosidase activity from both promoters in the absence of EF2638 (Fig. 2), consistent with the hypothesis that EF2638 functions as a repressor in *E. faecalis*.

Redox homeostasis is impaired in the Δ*EF2638* mutant. Rex factors modulate the expression of NADH-consuming enzymes in response to the redox poise to help maintain redox homeostasis in the cell. Because we observed derepression of genes encoding nu-

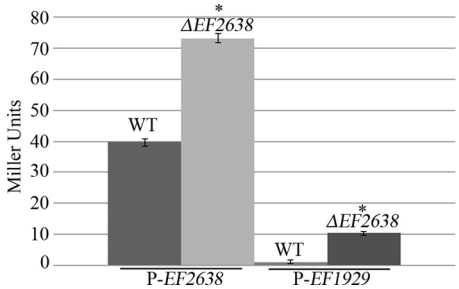

FIG 2 Assay for β-galactosidase activity. Cultures of plasmid-bearing OG1 (wild type) and DV87-4 (Δ*EF2638*) *E. faecalis* strains were grown in MH broth supplemented with erythromycin (10 μg/ml). Samples were collected in triplicate, and β-galactosidase activity was assayed as described in Materials and Methods. P-*EF2638*, putative EF2638 promoter driving the expression of promoterless *lacZ*; P-*EF1929*, EF1929-27 putative promoter driving the expression of promoterless *lacZ*. The data represent the means ± standard errors from two independent experiments. *, $P < 0.05$ versus wild type.

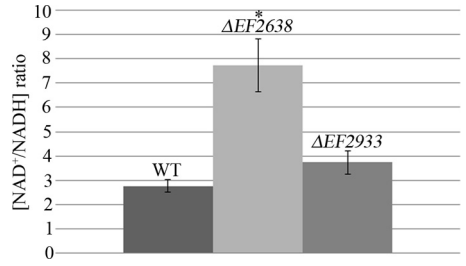

FIG 3 The Δ*EF2638* mutant exhibits an elevated NAD^+/NADH ratio. Wild-type OG1 (WT), DV87-4 (Δ*EF2638*), and DV122-1 (Δ*EF2933*) *E. faecalis* strains were grown in MH broth to exponential phase. Dinucleotides were extracted and the NAD^+/NADH ratio was measured as described in Materials and Methods. Data represent the means ± standard errors from at least two independent experiments. *, $P < 0.05$ versus wild type.

merous NADH-consuming enzymes in our transcriptome analysis of the Δ*EF2638* mutant, we reasoned that the NAD^+/NADH balance would be altered in the mutant. To test this, we determined the ratio of NAD^+ to NADH in exponentially growing cells using a dinucleotide cycling assay (Fig. 3). As would be expected upon overexpression of NADH-consuming enzymes, the NAD^+/NADH ratio was elevated in the Δ*EF2638* mutant compared to that in the wild type, supporting the hypothesis that EF2638 helps to maintain NAD^+/NADH homeostasis in *E. faecalis*.

To evaluate if the second putative *E. faecalis* Rex factor, EF2933, also plays a role in control of NAD^+/NADH homeostasis, we constructed an in-frame deletion mutant lacking EF2933 in an otherwise wild-type *E. faecalis* strain (OG1). Measurement of the NAD^+/NADH ratio revealed that the Δ*EF2933* mutant exhibited only a slightly elevated ratio (not statistically significantly different from that of the wild type), suggesting that the contribution of EF2933 to redox homeostasis is relatively minor compared to that of EF2638, at least under the growth conditions we used.

Aerobiosis impairs growth of the Δ*EF2638* mutant. During routine experiments with aerobically incubated (i.e., shaking) liquid cultures, we observed that growth of the Δ*EF2638* mutant was noticeably slower than that of the wild type. Careful analysis of the growth kinetics of the mutant (Fig. 4A) revealed that the Δ*EF2638* mutant exhibited a growth defect relative to that of the wild type when cultured with aeration. The defect was largely absent if the cultures—which were otherwise identical—were held static during growth (Fig. 4B), suggesting that the Δ*EF2638* mutant experiences, or is unable to cope with, elevated levels of oxidative stress that occur as a result of aeration. The growth defect of the mutant in aerated cultures could be complemented by introduction of *EF2638* with its native promoter at an ectopic locus in the chromosome of the Δ*EF2638* mutant (Fig. 4A), indicating that the defect is indeed due to loss of EF2638 function.

Although the specific source of intracellular reactive oxygen species in the above-described experiment has not been established, certain metabolic pathways in *E. faecalis* are known to produce reactive oxygen species directly as a by-product. For example, in the presence of O_2, *E. faecalis* can metabolize glycerol via the *glpK* pathway (38, 39), which includes *glpO* (glycerol-3-P oxidase) that converts glycerol-3-P to dihydroxyacetone-P with concomitant release of H_2O_2. Hypothesizing that such oxidative stress would be especially inhibitory for the Δ*EF2638* mutant, we evaluated growth in the presence of glycerol. We found that the Δ*EF2638* mutant was substantially impaired at growth on glycerol (Fig. 4C and D), even when cultures were incubated statically, consistent with the hypothesis that the Δ*EF2638* mutant is impaired at mounting an effective oxidative stress response. The observation that the Δ*EF2638* mutant was inhibited in glycerol cul-

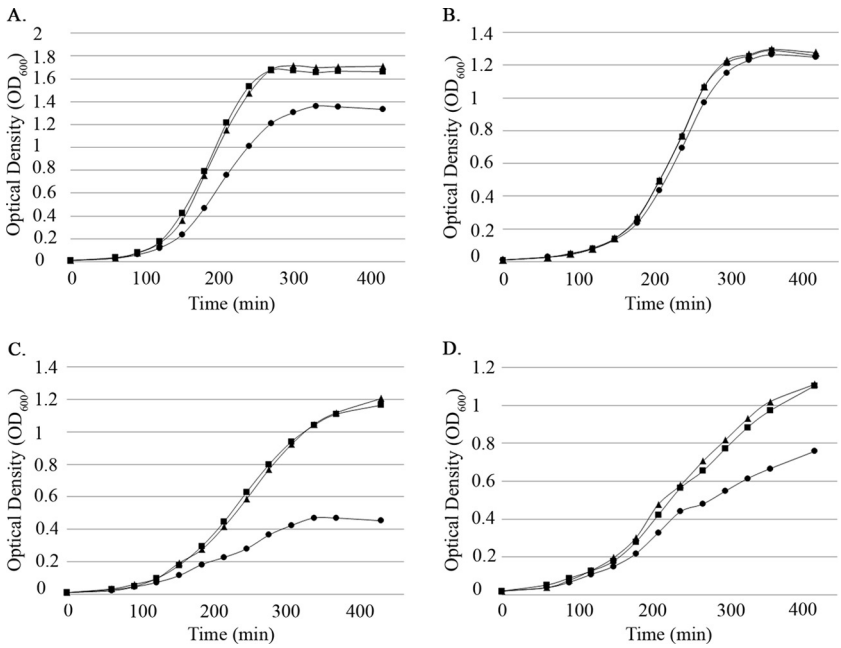

FIG 4 The Δ*EF2638* mutant exhibits a growth defect when grown with aeration. Bacteria were grown in MM9YE supplemented with 0.3% glucose (A and B) or 0.3% glycerol (C and D), and optical density was measured every 30 min at 600 nm. Cultures were grown with aeration (225 rpm, 37°C) (A, C) or statically (B, D). Wild type (OG1), squares; Δ*EF2638* (DV87-4) mutant, circles; Δ*EF2638* (*EF1116-EF1117)2*::*EF2638-3* (DV106) mutant, triangles. Representative results from at least two independent experiments are shown.

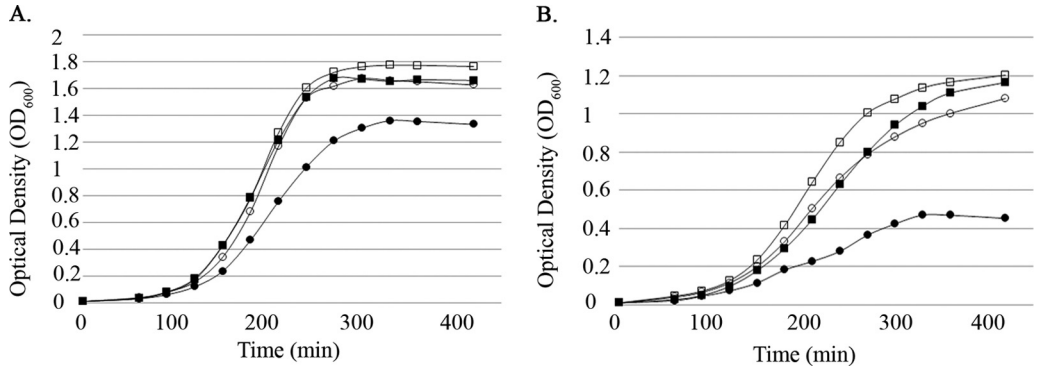

FIG 5 Catalase rescues the growth defect of the Δ*EF2638* mutant. Bacteria were grown with aeration (37°C, 225 rpm) in MM9YE containing 0.3% glucose (A) or 0.3% glycerol (B) with or without ~500 U/ml bovine catalase (open symbols, + catalase; closed symbols, − catalase), and optical density was measured every 30 min at 600 nm. Wild type (OG1), squares; Δ*EF2638* (DV87-4) mutant, circles. Representative results from at least two independent experiments are shown.

tures even under static conditions suggested that the *glpKOF* operon was being expressed at aberrantly high levels in the Δ*EF2638* mutant, leading to efficient H_2O_2 production even without the use of aeration to promote diffusion of oxygen into the culture.

H_2O_2 is responsible for the growth defect of the Δ*EF2638* mutant. The growth analyses described above suggested that H_2O_2, specifically, could be the proximal cause of the growth defect exhibited by the Δ*EF2638* mutant. To test this hypothesis, we supplemented the growth medium with bovine catalase to detoxify H_2O_2. Bovine catalase substantially improved the growth of the Δ*EF2638* mutant in aerated cultures of both glucose- and glycerol-containing media to levels comparable to those of the wild type (Fig. 5A and B), indicating that H_2O_2 is a significant cause of oxidative stress experienced by the Δ*EF2638* mutant. Of note, addition of bovine catalase also somewhat improved the growth of wild-type *E. faecalis* on glycerol, implying that H_2O_2 production during culture on glycerol is able to saturate the oxidative stress response of *E. faecalis* under the conditions used here. Supplementation of the growth medium with exogenous hematin (which is required for *E. faecalis* to produce an active, endogenously encoded catalase) also improved the growth of aerobically grown cells of the Δ*EF2638* mutant (see Fig. S2 in the supplemental material), consistent with the hypothesis that H_2O_2 is responsible for the growth defect.

Analogous growth studies of the Δ*EF2933* mutant also revealed an aeration-dependent growth defect (Fig. 6A and B), suggesting that the Δ*EF2933* mutant is also impaired at mounting an effective oxidative stress response. In contrast to the Δ*EF2638* mutant, however, the addition of catalase to the growth medium did not ameliorate the growth defect, indicating that the underlying cause of growth inhibition is likely to be different in the two Rex mutants.

Given that H_2O_2 appeared to be a major cause of the growth defect exhibited by the Δ*EF2638* mutant, we hypothesized that cultures of the Δ*EF2638* mutant would accumulate higher levels of H_2O_2. Because H_2O_2 rapidly equilibrates across the cytoplasmic membrane (40), we analyzed H_2O_2 levels in cell-free culture supernatants using an Amplex Red-based assay. Measurements of the levels of H_2O_2 in the supernatants of wild-type and Δ*EF2638 E. faecalis* strains revealed that H_2O_2 is indeed present at substantially elevated levels in cultures of the Δ*EF2638* mutant that have been subjected to aeration (Fig. 7; see also Fig. S3 in the supplemental material). In contrast, when cultures were incubated under static conditions (conditions in which there is no growth defect for the mutant; see Fig. 4), little difference was observed in culture supernatant H_2O_2 levels, consistent with the hypothesis that H_2O_2-derived oxidative stress is responsible for the growth defect of the Δ*EF2638* mutant. The Δ*EF2933* mutant did not exhibit a detectable increase in H_2O_2 accumulation compared to the wild type, consistent with the results of the growth studies (Fig. 6)

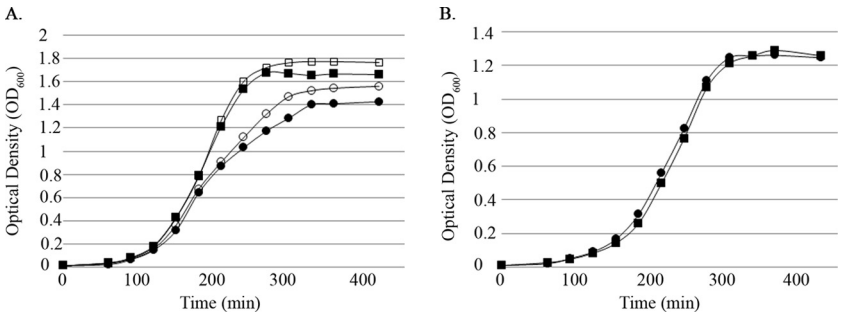

FIG 6 The Δ*EF2933* mutant exhibits an aeration-dependent growth defect that is not rescued by catalase. Bacteria were grown in MM9YE containing 0.3% glucose either with aeration (37°C, 225 rpm) (A) or statically (B). Aerated cultures were supplemented with catalase in some cases (open symbols, + catalase; closed symbols, − catalase). Optical density was measured every 30 min at 600 nm. Wild type (OG1), squares; Δ*EF2933* (DV122-1) mutant, circles. Representative results from at least two independent experiments are shown.

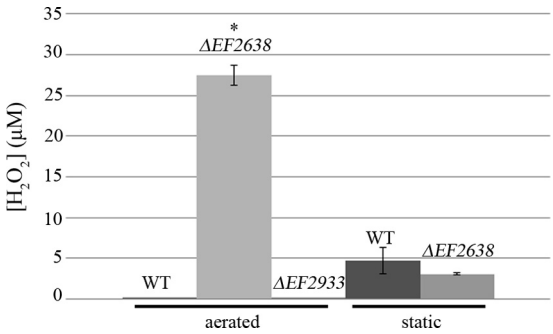

FIG 7 Accumulation of H_2O_2 by the Δ*EF2638* mutant. Wild-type OG1 (WT), DV87-4 (Δ*EF2638* mutant), and DV122-1 (Δ*EF2933* mutant) *E. faecalis* strains were grown to mid-log phase at 37°C with aeration (225 rpm) or statically in MM9YE supplemented with 0.3% glucose. Culture supernatants were collected and assayed for hydrogen peroxide using Amplex Red. Data are means ± standard errors from at least two independent experiments. *, $P < 0.05$ versus wild type.

in which catalase supplementation failed to improve growth of the Δ*EF2933* mutant.

DISCUSSION

As a natural inhabitant of the GI tract, *E. faecalis* must adapt to fluctuations in the levels of available oxygen and nutrients to maximize its competitive fitness. The mechanisms used by *E. faecalis* to achieve this are poorly understood. Changes in oxygen availability and nutrient metabolism can manifest themselves as changes in the redox state of the cell; for example, in *E. coli*, the steady-state NADH/NAD^+ ratio depends on the availability of suitable electron acceptors (1). The ratio is highest under anaerobic conditions and decreases when oxygen is introduced. In Gram-positive bacteria, the Rex transcription factor binds pyridine dinucleotides to monitor the redox state of the cell, thereby enabling Rex to modulate transcription of genes that are important for cellular redox homeostasis, including genes for alternative metabolic pathways, NADH reoxidation, and oxidative stress responses. The genome of *E. faecalis* encodes 2 putative Rex factors, EF2638 and EF2933, whose functions have not been described.

In this study, we explored the function of EF2638 in *E. faecalis* and found that it is indeed an authentic Rex factor. An *E. faecalis* mutant lacking EF2638 exhibited altered patterns of gene expression, primarily upregulation of genes expected to have critical roles in anaerobic metabolism and reoxidation of NADH, such as pyruvate-formate lyase, a putative fumarate reductase, and alcohol/aldehyde dehydrogenase (Table 2). Regulation of anaerobic gene expression has been described previously for Rex homologs in both *S. aureus* and *B. subtilis* (2, 35, 36). A palindromic consensus sequence (TGTGANNNNNNTCACA) for the *S. aureus* Rex binding site (the "Rex box") has been described (41), and inspection of the intergenic DNA sequences upstream of many of the genes that are differentially expressed in the Δ*EF2638* mutant revealed potential Rex box binding motifs (allowing for 1 or 2 mismatches), including EF0900, EF1613, EF0255, EF1225, and EF2933. We also identified a putative Rex box upstream of EF2638 itself. Mehmeti and coworkers (37) reported the presence of putative Rex boxes upstream of EF1613, EF0255, EF0900, and EF2933 but did not investigate a role for EF2638 in binding to these sites. We found that purified EF2638 was able to bind to several promoter fragments *in vitro* (Fig. 1), indicating that at least in some cases the effect of EF2638 on transcription is likely mediated by direct interaction with the promoters. Analysis of operon fusions to *lacZ* revealed that two of these promoters are more active *in vivo* in the Δ*EF2638* mutant (Fig. 2), indicating that increased transcriptional initiation (rather than reduced transcript degradation) is responsible for the elevated transcript abundance in the Δ*EF2638* mutant as detected by microarray analysis. Furthermore, DNA binding activity was reduced specifically in the presence of NADH, as has been observed for other Rex factors (4–7), suggesting that EF2638 likely monitors the NAD^+/NADH ratio *in vivo* to control transcription of genes in its regulon. Consistent with this hypothesis, Mehmeti and coworkers (37) also observed that expression of EF0900, EF0255, and EF1613 is upregulated under physiological growth conditions which are expected to result in elevated levels of NADH. In addition, our direct measurements of the NAD^+/NADH ratio demonstrated that the redox balance of the cell is substantially perturbed in the absence of EF2638 (Fig. 3), consistent with the hypothesis that a critical physiological function of EF2638 is to maintain redox homeostasis. We also note that the second putative Rex factor encoded in the *E. faecalis* genome, EF2933, was upregulated in the Δ*EF2638* mutant, suggesting an interconnected regulatory network. The details of this regulatory network remain to be elucidated, including whether or not EF2933 functions as an authentic Rex factor in *E. faecalis*, but our results clearly establish that EF2638 and EF2933 are not entirely functionally redundant. Indeed, the properties of mutants lacking either EF2638 or EF2933 are quite different from each other: while the Δ*EF2638* mutant exhibits an altered NAD^+/NADH ratio and accumulates aberrantly high levels of H_2O_2, the Δ*EF2933* mutant shares neither of those traits. Furthermore, although both mutants exhibit a growth defect when cultivated with aeration, only the Δ*EF2638* mutant can be rescued by the addition of catalase to scavenge H_2O_2. Further work is therefore required to define the function of EF2933 in *E. faecalis*.

Unexpectedly, we found that the Δ*EF2638* mutant accumulates substantially larger amounts of H_2O_2 than the otherwise isogenic wild-type *E. faecalis* when cultured under conditions of aeration (Fig. 7), and this H_2O_2 accumulates to sufficiently high concentrations to impose a growth defect on the mutant (Fig. 4). Although H_2O_2 is well known to participate in Fenton chemistry in cells to yield highly reactive and toxic hydroxyl radicals, we suspect that H_2O_2 itself is the primary cause of the cellular damage leading to growth inhibition of the Δ*EF2638* mutant. This suggestion is based on our observation that inclusion of an iron chelator (250 μM 2′2 bipyridyl) in the medium to prevent iron-catalyzed Fenton reactions did not improve growth of the Δ*EF2638* mutant under conditions of aeration (not shown), although we cannot unequivocally exclude the possibility that bipyridyl does not penetrate *E. faecalis* cells efficiently. More work will be required to identify the biological targets that are damaged by H_2O_2 to manifest the growth defect observed here.

Why does H_2O_2 accumulate in the Δ*EF2638* mutant? It seems possible that the mutant might be generating more H_2O_2 under conditions of aeration than the wild-type strain. H_2O_2 production in *E. coli* can occur as a result of adventitious autoxidation by oxygen of flavin-containing enzymes, including fumarate reductase (42). Our microarray data indicate that genes encoding functions likely relevant under anaerobic conditions, such as a putative fumarate reductase, as well as other NADH-consuming dehydro-

genases, are expressed at aberrantly high levels in the Δ*EF2638* mutant, when they would normally be repressed. Elevated levels of these enzymes might offer increased opportunity for adventitious autoxidation to occur when the Δ*EF2638* mutant is cultured with aeration, thereby increasing the rate of H_2O_2 production. Alternatively, the H_2O_2 detoxification systems of the Δ*EF2638* mutant might be impaired. *E. faecalis* encodes an NADH peroxidase (*npr*) that uses NADH to directly reduce H_2O_2 to H_2O (17). NADH peroxidase is known to be an important defense in *E. faecalis* against both exogenously added and endogenously produced H_2O_2 under a variety of growth conditions, and a mutant lacking *npr* accumulates H_2O_2 in the growth medium at an enhanced rate (43). The reduced availability of NADH in the Δ*EF2638* mutant (Fig. 3) might starve Npr of reducing power needed for the enzyme to function efficiently, leading to accumulation of aberrantly high levels of H_2O_2. It remains less clear what the relevant source of reducing power *in vivo* is for the other two known *E. faecalis* peroxidases (alkyl hydroperoxide reductase and thiol peroxidase), but one can imagine that the redox imbalance present in the Δ*EF2638* mutant could indirectly impair the ability of these enzymes to function as well. In any case, the relative importance of the three *E. faecalis* peroxidases has been reported to vary depending on the growth conditions, such as the available carbon source, and certainly the proximal mechanism(s) responsible for accumulation of H_2O_2 under a particular set of environmental conditions might be expected to vary in a similar fashion.

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