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Enterococcus faecalis Manganese Exporter MntE Alleviates Manganese Toxicity and Is Required for Mouse Gastrointestinal Colonization

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ABSTRACT Bacterial pathogens encounter a variety of nutritional environments in the human host, including nutrient metal restriction and overload. Uptake of manganese (Mn) is essential for *Enterococcus faecalis* growth and virulence; however, it is not known how this organism prevents Mn toxicity. In this study, we examine the role of the highly conserved MntE transporter in *E. faecalis* Mn homeostasis and virulence. We show that inactivation of *mntE* results in growth restriction in the presence of excess Mn, but not other metals, demonstrating its specific role in Mn detoxification. Upon growth in the presence of excess Mn, an *mntE* mutant accumulates intracellular Mn, iron (Fe), and magnesium (Mg), supporting a role for MntE in Mn and Fe export and a role for Mg in offsetting Mn toxicity. Growth of the *mntE* mutant in excess Fe also results in increased levels of intracellular Fe, but not Mn or Mg, providing further support for MntE in Fe efflux. Inactivation of *mntE* in the presence of excess iron also results in the upregulation of glycerol catabolic genes and enhanced biofilm growth, and addition of glycerol is sufficient to augment biofilm growth for both the *mntE* mutant and its wild-type parental strain, demonstrating that glycerol availability significantly enhances biofilm formation. Finally, we show that *mntE* contributes to colonization of the antibiotic-treated mouse gastrointestinal (GI) tract, suggesting that *E. faecalis* encounters excess Mn in this niche. Collectively, these findings demonstrate that the manganese exporter MntE plays a crucial role in *E. faecalis* metal homeostasis and virulence.

KEYWORDS *Enterococcus faecalis*, gastrointestinal infection, manganese export, metal homeostasis

Manganese (Mn) is an essential metal for most bacteria and serves as a cofactor for proteins involved in metabolism, DNA replication, respiration, and oxidative stress (1). Accordingly, Mn acquisition contributes to bacterial virulence in multiple bacterial species (2–4). In order to limit bacterial growth and virulence, the host sequesters Mn as a defense response termed nutritional immunity (1, 4–6). To counteract these host-mediated defenses, many bacterial pathogens, including *Enterococci*, possess dedicated systems to acquire Mn.

Bacteria possess conserved ABC and NRAMP-family transporters for manganese uptake (1, 2). In *Enterococcus faecalis*, three manganese uptake systems have been described—the ABC-type transporter encoded by *efaCBA* and two NRAMP transporters encoded by *mntH1* and *mntH2* (7). These three Mn transport systems are functionally redundant since deletion of all three transporter systems (*efaCBA*, *mntH1*, and *mntH2*) is required to abrogate intracellular Mn accumulation, rendering the triple mutant

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severely impaired in growth (7). Furthermore, deletion of both *efaCBA* and *mntH2* or all three transporters together results in attenuated colonization in rabbit endocarditis and mouse catheter-associated urinary tract infection (CAUTI) models (7). Together, these observations demonstrate that the ability to acquire Mn is essential for *E. faecalis* virulence.

In contrast to Mn influx mechanisms that have been characterized in *E. faecalis*, Mn export pathways have not been described. The contribution of Mn export to bacterial pathogenesis and virulence has been demonstrated for some bacterial species (8–15) and is dependent on two widely characterized classes of exporters—MntE, a cation diffusion facilitator (CDF) family protein, and MntP, a 6-transmembrane helix protein typical of LysE transporter family members (8). In *Escherichia coli* and *Neisseria meningitidis*, deletion of *mntP* and *mntX* Mn exporters, respectively, both of which belong to the MntP class of exporters, results in elevated intracellular Mn and increased sensitivity to Mn toxicity (16–18). In the case of *N. meningitidis*, loss of MntX results in reduced bacterial titers recovered from the serum in a mouse sepsis model (17). The CDF family of proteins has been characterized in several bacterial species, where they display broad metal specificity (19–23). In *Streptococcus pneumoniae* and *Streptococcus pyogenes*, deletion of *mntE* results in increased sensitivity to Mn toxicity and intracellular Mn accumulation (9, 10, 24). Similarly, in *Streptococcus suis*, deletion of *mntE* results in increased sensitivity to Mn toxicity and, like in *S. pyogenes*, displays increased sensitivity to oxidative stress (10, 12). Additionally, MntE mutants in *S. pneumoniae* and *S. suis* are attenuated in mouse models of infection (9, 12). In *Staphylococcus aureus*, an *mntE* mutant displays increased sensitivity to Mn and oxidative stress when grown in Mn-enriched medium and accumulates intracellular Mn (25). Furthermore, loss of *S. aureus mntE* results in reduced mortality in mice after retro-orbital infection (25). Taken together, these reports indicate that Mn import, export, and homeostasis are important for virulence in multiple pathogens.

In a prior study, we discovered that a putative cation efflux transporter, *OG1RF_10589* (*OG1RF_RS03085*), contributed to *E. faecalis* biofilm formation specifically in iron (Fe)-supplemented medium (26). Previous reports showed that *E. faecalis* *OG1RF_10589* (identified as *czcD* in those studies) transcription was downregulated when grown in blood (27) and subsequently induced when *E. faecalis* was grown planktonically in both Fe-supplemented (28) and Mn-supplemented medium (29). The goal of this study was to characterize the function of the predicted cation efflux transporter *OG1RF_10589*. We show that this protein functions in Mn efflux and hence renamed the gene *mntE*. An *E. faecalis mntE* mutant grown in excess Mn accumulates intracellular Mn and Fe but is selectively sensitive only to Mn and not Fe toxicity. However, when *E. faecalis* biofilms were grown in Fe-supplemented medium, the conditions in which *mntE* contributed to augmented biofilm formation, only three genes were differentially regulated in the *mntE* mutant compared to the wild type; the glycerol catabolic genes (*glpF2*, *glpK*, and *glpO*) were all upregulated. Since we show that glycerol supplementation also promotes biofilm growth, this result suggests that upregulation of glycerol catabolic genes likely contributes to the enhanced biofilm growth of the *mntE* mutant in iron-supplemented medium that we reported previously. Finally, we demonstrate that MntE contributes to colonization of the mouse gastrointestinal (GI) tract, suggesting that maintaining MntE-mediated metal homeostasis confers a fitness advantage to *E. faecalis* in the mammalian host.

RESULTS

***mntE* is required for planktonic growth and biofilm formation when Mn is in excess.** The *Enterococcus faecalis* OG1RF genome encodes a putative cation efflux transporter (*OG1RF_10589*), and this translated gene product displays 69% and 80% amino acid similarity to the zinc exporter CzcD (GenBank accession no. [CW193218.1](#)) and the manganese exporter MntE (*spy1552*), respectively, in *Streptococcus pneumoniae* (9, 10) (see Materials and Methods). MntE belongs to the cation diffusion facilitator (CDF) family of metal efflux pumps (30). The predicted *E. faecalis* cation efflux trans-

porter *OG1RF_10589* also shares 25% amino acid identity with the Mn exporter MneP (formerly called YdfM) in *Bacillus subtilis* and 25% identity with FieF in *E. coli*, which export Mn and Fe, respectively (22, 31). Pairwise alignment of *E. faecalis* MntE showed higher similarity to *S. pneumoniae* and *E. coli* compared to *B. subtilis* (see Fig. S1 in the supplemental material). *E. faecalis* *OG1RF_10589* possesses a DXXXD motif in transmembrane domain 5 (starting at amino acid 171 in the predicted OG1RF protein) (Fig. S2), which is typical of CDF transporters (32). CDF transporters possess six putative transmembrane (TM) domains, a signature N-terminal amino acid sequence, and a characteristic C-terminal cation efflux domain (33, 34). The experiments below establish that *OG1RF_10589* shares properties with MntE and functions in Mn homeostasis, so we refer to *OG1RF_10589* as *mntE* henceforth. Based on the amino acid identity between *E. faecalis* *OG1RF_10589* and Mn transporters in other bacterial species, as well as transcriptional induction of the gene in the presence of both Mn and Fe (28, 29), we hypothesized that *E. faecalis* *OG1RF_10589* (MntE) exports Mn and Fe and that the absence of this gene would result in increased sensitivity to metal toxicity and decreased growth in the presence of excess Mn and/or Fe. To test this prediction, we performed planktonic growth assays comparing wild-type *E. faecalis* *OG1RF* to an isogenic *mntE::Tn* mutant in increasing concentrations of Mn, Fe, zinc (Zn), copper (Cu), and magnesium (Mg). We observed a dose-dependent reduction in growth of the *mntE::Tn* mutant when grown in Mn-supplemented medium after 8 h of incubation as measured by optical density (Fig. 1A). However, there was no growth defect for the *mntE::Tn* mutant when the medium was supplemented with any other cationic metal (Fig. S3). Similarly, we observed significantly fewer CFU (approximately 2- to 3-log reduction) of *mntE::Tn* after 6 h for all Mn concentrations tested (Fig. 1B). Complementation of the *mntE::Tn* mutant with *mntE* under the control of a nisin-inducible promoter on a plasmid rescued Mn-mediated growth inhibition and restored the CFU to near wild-type levels after 8 h of exposure to 2 mM Mn (Fig. 1C and D).

Since the absence of *mntE* leads to Mn-mediated growth inhibition under planktonic conditions (Fig. 1A), we next tested whether this was the case for biofilm formation. To address this, we performed static *in vitro* crystal violet (CV) biofilm assays and macrocolony biofilm assays. With this biofilm accumulation assay, wild-type *E. faecalis* biofilm biomass was not significantly altered in the presence of 2 mM Mn. In contrast, the *mntE::Tn* mutant was attenuated for biomass accumulation (Fig. 1E). Similarly, in biofilm macrocolony assays, biofilm CFU were not affected when wild-type *E. faecalis* biofilm biomass was grown in 2 mM Mn, but the *mntE::Tn* mutant had significantly fewer biofilm-associated CFU when grown in excess Mn (Fig. 1F). Complementation of *mntE::Tn* with *mntE* in *trans* restored biofilm CFU to wild-type levels. These results demonstrate that the absence of *mntE* leads to increased sensitivity to Mn during both planktonic and biofilm growth.

Absence of *mntE* results in intracellular metal accumulation. The ability to regulate intracellular Mn is a key determinant for cell survival and growth. Based on its predicted function in Mn export, we hypothesized that the absence of *mntE* would lead to increased intracellular Mn. To test this hypothesis, we performed inductively coupled plasma mass spectrometry (ICP-MS) on cells isolated from static 24-h biofilms grown in 2 mM Mn-supplemented medium. While we did not observe differences in intracellular metal accumulation when the *mntE::Tn* mutant was grown in control medium (Fig. 2A), we observed that wild-type *E. faecalis* accumulated more intracellular Mn when grown in Mn-supplemented medium, and the *mntE::Tn* mutant accumulated significantly more intracellular Mn compared to the wild type when both were grown in 2 mM Mn-supplemented medium (Fig. 2B). Complementation of the *mntE::Tn* mutant with *mntE* restored intracellular Mn levels of the *mntE::Tn* mutant to that of the wild-type empty vector control strain (Fig. 2B). Notably, growth of the *mntE::Tn* mutant in Mn-supplemented medium also resulted in 10-fold more intracellular Mg and 30-fold more intracellular Fe compared to the wild-type strain (Fig. 2B). We previously showed that the absence of *mntE* resulted in enhanced biofilm growth in iron-supplemented

FIG 1 MntE is necessary for planktonic and biofilm growth under Mn stress. (A to D) Optical density measurement of planktonic growth in Mn-supplemented medium over the course of 8 h (A and C) and the corresponding CFU enumerated at 6 h and 8 h (B and D). (Continued on next page)

FIG 2 Intracellular manganese, iron, and magnesium content in *E. faecalis* biofilm. ICP-MS analysis of intracellular metals of (A) 24-h biofilms grown in control medium, (B) 2 mM Mn-supplemented medium, and (C) 2 mM Fe-supplemented medium. Data points represent nine independent biological replicates assessed in 3 experiments. Statistical analysis was performed using two-way ANOVA with the Bonferroni multiple-comparison test. The error bar represents the SEM. WT, wild type. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

medium (26). If MntE also exports Fe, as the data in Fig. 2B suggest, the absence of *mntE* should give rise to increased intracellular Fe due to intracellular accumulation. Indeed, we observed that the *mntE::Tn* grown in Fe-supplemented medium accumulated significantly more intracellular Fe compared to the wild type, whereas intracellular Mn and Mg were unchanged compared to the wild type (Fig. 2C). Complementing the mutant strain with *mntE* resulted in restoration of intracellular Fe to levels observed in the wild-type empty vector control strain (Fig. 2C). These findings suggest that MntE has the capacity to export both Mn and Fe.

Magnesium supplementation alleviates manganese-mediated growth inhibition. Since we observed increased intracellular Mg in the *mntE::Tn* biofilms grown in Mn-supplemented medium (Fig. 2), we reasoned that increasing intracellular Mg may

FIG 1 Legend (Continued)

D). For optical density measurement, statistical analysis was performed at the 8 h time point using one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison test. For CFU enumeration, statistical analysis was performed using two-way ANOVA with the Bonferroni multiple-comparison test. Data points represent 3 experiments, with three independent biological replicates averaged in each experiment. (E) *E. faecalis* biofilm biomass quantification grown for 120 h using crystal violet staining. (F) CFU enumeration of macrocolony biofilms. For both biofilm biomass quantification and macrocolony enumeration, statistical analysis was performed using two-way ANOVA with the Bonferroni multiple comparison test. Data points represent at least 3 experiments, with three independent biological replicates averaged in each experiment. The error bar represents the standard error of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

be a bacterial response to counter accumulated intracellular Mn-mediated toxicity as reported for *Bradyrhizobium japonicum* (35). Therefore, we tested if supplementation of Mg would restore growth attenuation of *mntE::Tn* when grown in 2 mM Mn-supplemented medium during planktonic and biofilm growth. Indeed, addition of Mg to Mn-supplemented medium restored growth to the *mntE::Tn* mutant and promoted growth of wild-type *E. faecalis* in a dose-dependent manner (Fig. 3A). However, in the biofilm assay, we observed significantly attenuated biofilm formation with increasing Mg supplementation for the wild type, whereas biofilm of the *mntE::Tn* mutant was augmented with Mg supplementation (Fig. 3B), as it was observed similarly during planktonic growth (Fig. 3A). In the macrocolony assay, the 1-log reduction in CFU observed for the *mntE::Tn* mutant in 2 mM Mn-supplemented medium was similarly restored at all concentrations of Mg tested (Fig. 3C). Alleviation of Mn-mediated growth inhibition of *mntE::Tn* was specific to Mg, since Fe supplementation did not restore growth (Fig. S4). Furthermore, 2 mM Mg added to Mn-supplemented medium resulted in reduced intracellular Mn for both wild-type *E. faecalis* (3.33-fold) and *mntE::Tn* (2.21-fold) (Fig. 3D). In contrast, supplementing Mn-supplemented medium with 0.5 mM Mg resulted in 2-fold and 4-fold increased intracellular Mg in the wild type and the *mntE::Tn* mutant, respectively. However, in both the wild type and the *mntE::Tn* mutant, 2 mM Mg supplementation to the same Mn-supplemented medium resulted in the reversion of intracellular Mg levels to when Mn was supplemented alone (Fig. 3E). Together, these observations suggest that Mg supplementation rescues Mn-mediated toxicity and growth inhibition in *E. faecalis* and that Mg accumulation can impact intracellular Mn pools and modulate biofilm growth.

***mntE* expression is manganese responsive in *E. faecalis* biofilm.** Since complementation of *mntE* alleviates intracellular Mn accumulation in the *mntE::Tn* mutant, we hypothesized that *mntE* would be transcriptionally upregulated upon *E. faecalis* biofilm growth in Mn-supplemented medium, as previously described for planktonically grown *E. faecalis* (29). We performed reverse transcription-quantitative PCR (qRT-PCR) to analyze *mntE* transcript levels and observed a significant increase in expression for wild-type *E. faecalis* biofilms grown in Mn-supplemented medium compared to normal growth medium (Fig. 4). Since Mn exposure resulted in upregulation of the *S. pneumoniae* MntE exporter and pilus expression (9), and since pilus expression is critical for *E. faecalis* biofilm formation (36, 37), we hypothesized that pilus expression might be Mn-responsive in *E. faecalis* biofilms as well. Indeed, we observed that *ebpC*, encoding the major subunit of the *E. faecalis* endocarditis and biofilm-associated pilus (Ebp) (36), was also significantly induced in Mn-supplemented medium (Fig. 4).

Although the *mntE::Tn* mutant accumulates intracellular Fe, and *mntE* is induced in response to Fe during planktonic growth (28), *mntE* is not upregulated in *E. faecalis* biofilms when grown in Fe-supplemented medium (data not shown). However, we previously showed that the absence of *mntE* resulted in augmented biofilm formation in Fe-supplemented medium (26). To identify other Fe-regulated genes that might contribute to Fe-augmented biofilm formation, we performed RNA sequencing and compared transcriptional profiles of wild-type and *mntE::Tn* *E. faecalis* biofilms grown in Fe-supplemented medium. Strikingly, the only differentially regulated genes were the upregulation of glycerol catabolic genes (*glpF2*, *glpO*, and *glpK*) in the *mntE* mutant in response to Fe compared to the non-iron-supplemented medium TSBG (tryptic soy broth supplemented with 25% [vol/vol] D+ glucose) control (Table S1). We speculated that glycerol serves as an energy source to promote biofilm growth for the *mntE::Tn* mutant in Fe-supplemented medium. We were unable to simultaneously delete *mntE* together with any of the glycerol catabolic genes in order to test this hypothesis. Instead, increasing glycerol concentrations in the growth medium enhanced biofilm formation in both the wild-type control and the *mntE::Tn* mutant, regardless of Fe supplementation (Fig. S5). In contrast, these three glycerol catabolic genes (*glpF2*, *glpO*, and *glpK*) were not significantly upregulated under Mn-supplemented conditions in wild-type *E. faecalis* biofilm (Fig. 4), and global transcriptional analysis showed that

FIG 3 Magnesium supplementation rescues manganese-mediated growth inhibition. (A) Planktonic growth kinetics. Data points represent 3 experiments, with three independent biological replicates averaged in each experiment. (B) *E. faecalis* biofilm biomass (Continued on next page)

FIG 4 *mntE* expression is induced in *E. faecalis* biofilm upon manganese supplementation. qRT-PCR of *E. faecalis* OG1RF biofilm grown in 2 mM manganese. Data points represent 2 experiments, with three independent biological replicates averaged in each experiment. Statistical analysis is performed using one-way ANOVA with the Fisher LSD test. The error bar represents SEM. *, $P < 0.05$; **, $P < 0.01$.

these genes are not Fe responsive in wild-type OG1RF biofilm grown in Fe-supplemented medium (38). Taken together, these results indicate that upregulation of glycerol catabolic genes is specifically observed in the absence of *mntE* when intracellular Fe levels are high and that glycerol supplementation contributes to biofilm growth.

Absence of MntE does not alter oxidative stress tolerance in *E. faecalis*. Since the absence of *mntE* results in intracellular Mn accumulation, we speculated that accumulation of Mn may alter *E. faecalis* oxidative stress tolerance. The increased availability of intracellular Mn could enhance Mn-dependent antioxidant defenses, as has been reported for *Streptococcus* spp. (10, 12). Alternatively, increased intracellular Mn could lead to increased sensitivity to oxidative stress as reported for *Xanthomonas oryzae* and *S. pyogenes* (8, 12, 13). However, the *E. faecalis mntE::Tn* mutant did not display altered sensitivity to oxidizing agents compared to the wild type (Fig. S6A and B), nor did hydrogen peroxide production significantly change compared to the wild type, as has been reported for *S. pneumoniae* when Mn is in excess (9) (Fig. S6C). Therefore, we conclude that increased intracellular Mn does not impact oxidative stress tolerance in *E. faecalis*.

MntE is required for *E. faecalis* expansion in the mouse GI tract. Given the importance of Mn acquisition for *E. faecalis* virulence (7) and the role of MntE in Mn homeostasis, we tested whether MntE contributes to *E. faecalis* virulence. Using an antibiotic-treated mouse model of gastrointestinal (GI) tract colonization, we observed that the *mntE::Tn* mutant was significantly attenuated for colonization in the cecum, small intestine, and colon compared to wild-type *E. faecalis* (Fig. 5). By contrast, the *mntE::Tn* mutant survived as well as wild-type *E. faecalis* in the drinking water (data not shown), and this indicates that the absence of MntE did not affect overall growth fitness, which could potentially result in the attenuated colonization observed. These results suggest that the GI tract represents a natural reservoir abundant with Fe, Mn, or other likely MntE-effluxed metals. Indeed, ICP-MS analysis of GI tissue showed the presence of Mn and Fe (Fig. S7). Therefore, our results demonstrate that MntE and Mn homeostasis, and potentially MntE-mediated Fe homeostasis, are important for *E. faecalis* colonization of the antibiotic-treated mouse GI tract.

DISCUSSION

We previously showed that the absence of MntE resulted in enhanced iron-augmented biofilm (26); however, the role of MntE and its contribution to iron-

FIG 3 Legend (Continued)

quantification grown for 120 h using crystal violet staining. (C) CFU enumeration of macrocolony biofilms. For both biofilm biomass quantification and macrocolony enumeration, data points represent nine independent biological replicates assessed in 3 experiments. For both biofilm biomass quantification and macrocolony CFU enumeration, statistical analysis was performed using the unpaired *t* test with Welch's correction and two-way ANOVA with the Bonferroni multiple-comparison test respectively. (D) ICP-MS analysis of intracellular manganese. (E) ICP-MS analysis of intracellular magnesium. For ICP-MS analysis of intracellular manganese and magnesium, data points represent six independent biological replicates assessed in 2 experiments. Statistical analysis was performed using one-way ANOVA with the Bonferroni multiple-comparison test. Error bar represents the SEM. WT, wild type. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

FIG 5 MntE is required for colonization in the mouse gastrointestinal tract. Bacterial CFU in GI tissues. Data points represent the CFU recovered from each mouse (for the wild type [WT], $n = 30$; for the *mntE::Tn* mutant, $n = 30$) collected in three independent experiments. Statistical significance was determined by Mann-Whitney test. The black line indicates the median. The dotted line indicates the limitation of detection at a CFU of 40. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

augmented biofilm were not characterized. We show here that MntE is essential for Mn homeostasis to prevent Mn toxicity and contributes to changes in intracellular Fe and Mg pools, which in turn, can alter glycerol catabolism and growth. Finally, we demonstrate that MntE is required for *E. faecalis* expansion in the mouse gastrointestinal (GI) tract.

Metal exporters serve to maintain intracellular metal homeostasis. The observation that Mg and Fe accumulate within *E. faecalis* *mntE* mutant cells during growth in Mn-supplemented medium suggests either that MntE can efflux Mg and Fe or that growth in Mn-supplemented medium results in the increased import of these metals. Although Mg²⁺ has similar ionic radii to Fe²⁺ and Mn²⁺, we are not aware of any reports documenting Mg²⁺ efflux from the CDF family of proteins (30) or Mg transporters in *E. faecalis*. However, conserved families of bacterial proteins have been identified for Mg uptake and efflux (39). Therefore, these findings together suggest that the observed Mg accumulation in the *E. faecalis* *mntE* mutant may be mediated by transporters which have yet to be characterized in *E. faecalis*. With regard to Fe accumulation, and consistent with the possibility that MntE effluxes Fe, *mntE* was previously reported to be upregulated when *E. faecalis* was grown in Fe-supplemented medium planktonically (28), and the absence of MntE resulted in increased intracellular Fe. While the absence of MntE also similarly resulted in intracellular Mn accumulation, no growth inhibition for the *mntE::Tn* mutant was observed even at the highest Fe concentration tested compared to Mn in this study. Based on these data, we speculate that MntE may be the sole exporter for Mn in *E. faecalis*, whereas MntE may be one of several redundant export systems for Fe.

Altered Mn homeostasis affects sensitivity to oxidative stress and has been demonstrated to attenuate virulence in *S. pyogenes* (10, 11), *S. pneumoniae* (9, 40), and *S. aureus* (25). Given the sequence and functional conservation between *E. faecalis* MntE and MntE from other Gram-positive species, we examined its role in *E. faecalis* oxidative stress tolerance and virulence. While we found no evidence for a role of *E. faecalis* MntE during growth in the presence of oxidative damaging agents, it contributes to growth in the antibiotic-treated GI tract. A major factor in *E. faecalis* adhesion, virulence, and biofilm formation is the sortase-assembled pilus (Ebp) (37, 41–43). However, transcription of the gene encoding the major pilus subunit *ebpC* is upregulated in Mn-supplemented medium, even when biofilm biomass accumulation is attenuated compared to the wild type. Since Ebp is important for GI colonization (44), and since the *mntE* mutant is attenuated in mouse GI colonization, we speculate that either the

upregulation of pilus expression observed *in vitro* does not occur in the mouse GI tract or that pilus expression *in vivo* is insufficient to complement the virulence defect of the *mntE* mutant.

In this study, we observed that when Mn is in excess, an *E. faecalis mntE* mutant accumulates intracellular Mn, Fe, and Mg. It is likely that altered intracellular metal homeostasis may be the driving force underlying Mn-mediated growth inhibition and the *in vivo* virulence defect. Multiple mismetallation outcomes could be at play, resulting in its sensitization to Mn toxicity. The accumulation of intracellular Mg in the absence of *mntE*, coupled with the ability of Mg supplementation to reduce intracellular Mn, restore growth, and protect from Mn toxicity, suggests that mismetallation of Mg-metalloproteins by Mn may be an underlying reason for *E. faecalis* growth inhibition. Magnesium can serve as a cofactor for Mg-dependent enzymes and can help to stabilize protein complexes and cellular structures (39, 45). Due to the similar ionic radii of these divalent ions, we postulate that Mn cations can displace Mg. The displacement of Mg could, in turn, result in nonfunctional or altered function of the metalloproteins. Although there is no evidence for this in *E. faecalis* to date, this idea has been proposed for other bacterial spp. In *B. subtilis*, the loss of *mpfA*, encoding a Mg efflux pump, leads to increased intracellular Mg and suppressed Mn toxicity (46). Despite the increased sensitivity to Mg toxicity, the *mpfA* mutant is less sensitive to Mn toxicity. Further, supplementing the growth medium with Mg rendered both wild-type *B. subtilis* and its Mn efflux mutant ($\Delta mneP\Delta mneS$) less sensitive to Mn intoxication and also less sensitive to Fe, Co, and Zn intoxication (46). In *Bradyrhizobium japonicum*, removal of Mn from Mg-limited medium partially restores growth defects due to depletion of Mg, which suggests that the presence of Mn under Mg-limited conditions is toxic to *B. japonicum* (35). Consistent with the speculation that Mn and Mg can competitively bind to metalloproteins and alter protein function, supplementing *B. japonicum* with either metal enhances the activity of Mg-dependent isocitrate dehydrogenase; in contrast, the addition of Mn inhibited Mg-dependent isocitrate lyase (35). Additionally, the activity of another *B. japonicum* Mg-dependent enzyme, 5-aminolevulinic acid (ALA) dehydratase, was 3-fold higher with Mn as a cofactor than with Mg (35). Despite the limited literature describing mismetallation, these findings suggest that mismetallation of Mg metalloproteins by Mn can alter enzymatic activity and affect growth, and this may be relevant for *E. faecalis*.

We speculate that the increased Fe levels observed in the *E. faecalis mntE* mutant may serve to maintain the intracellular Fe/Mn ratio necessary for cellular processes under Mn stress. Altered metal homeostasis occurs when bacteria are under Mn stress, whereby the accumulation of intracellular Mn is accompanied with changes in Fe and Cu levels, as described for multiple bacterial species. Previously we have also shown that increased intracellular Fe is accompanied by increased Cu levels when *E. faecalis* biofilm is grown in iron-supplemented medium (26). In *S. pneumoniae*, deletion of *mntE* results in increased intracellular Mn, and similarly, Fe and Cu intracellular levels are increased (24). Growth of the *S. pneumoniae mntE* deletion mutant under Mn stress resulted in upregulation of genes involved in both Fe and Cu uptake (9, 47, 48), and these observations are consistent with the intracellular accumulation of these metals (24). Similarly, in *E. coli*, overexpression of the Mn exporter encoded by *mntS*, or deletion of the Mn exporter encoded by *mntP*, resulted in elevated intracellular Mn (16, 18). However, overexpression of *mntS* resulted in decreased intracellular Fe, due to downregulation of Fur-regulated iron uptake genes (18, 49, 50). In the context of *E. coli*, it was proposed that Mn can substitute for Fe; thus, it is likely that Mn-bound Fur is a capable repressor for iron acquisition gene expression. In *S. aureus*, loss of *mntE* expression resulted in elevated intracellular Mn and reduced intracellular Fe (25). It has been suggested that the elevated intracellular Mn drives repression of the PerR regulon, which limits oxidative stress responses and Fur-dependent expression of iron acquisition systems in *S. aureus* (51, 52). Altogether, these reports demonstrate that bacteria tightly regulate intracellular Mn/Fe ratios, and altered homeostasis of these transition metals can alter gene transcription and growth. Therefore, it is likely that *E.*

faecalis employs similar strategies to regulate intracellular Fe/Mn ratios, and alteration of these ratios can impact global gene transcription. We speculate that inactivation of *mntE* did not greatly impact growth in normal medium or oxidative stress tolerance due to the presence of redundant antioxidant enzymes in *E. faecalis* (66). Future studies should focus on the transcriptional changes, including *fur* and *perR* regulons under altered intracellular Fe/Mn ratios due to deletion of *mntE*, and how these genes impact intracellular metal homeostasis.

To elucidate the mechanisms involved in enhancement of iron-augmented *E. faecalis* biofilm formation by an *mntE* mutant, we discovered that glycerol catabolic genes (*glpF2*, *glpO*, and *glpK*) were induced in the *mntE::Tn* mutant when grown in iron-supplemented conditions that also drive intracellular iron accumulation. It is unclear how iron might stimulate glycerol catabolism, but we do know that *E. faecalis* has two glycerol catabolic pathways, one of which is dependent on ATP-mediated phosphorylation of glycerol by glycerol kinase (GlpK) to yield glycerol-3-phosphate (glycerol-3-P) (53). Here, we propose a model in which upregulation of the glycerol importer (*glpF2*), alpha-glycerophosphate oxidase (*glpO*), and glycerol kinase (*glpK*) is driven by the presence of increased intracellular Fe. Consistent with this idea, an *E. faecalis* V583 *fur* deletion mutant is incapable of repressing iron uptake, and when grown in iron-supplemented medium, displayed significantly increased transcription of glycerol dehydrogenase and glycerol kinase (*glpK*) (54). The relationship between glycerol catabolism and Fe availability is unclear at this time. Since Fe may function as a biocatalyst for oxidation of glycerol (55) and is an important transition metal for microbial growth (56), we speculate that Fe positively impacts glycerol uptake, and the increased uptake of glycerol, which in turn is converted to glycerol-3-phosphate (G3P) in glycolysis, drives increased energy production and increased biofilm growth.

Collectively, these findings suggest that MntE is a Mn exporter. Since MntE is conserved across a number of Gram-positive and Gram-negative bacteria, we propose that this Mn efflux system is a common strategy for Mn homeostasis in bacteria. In *E. faecalis*, we establish that efflux of Mn is vital for growth and successful colonization in the gastrointestinal tract (GI) and that the Mn exporter MntE may be a promising target in developing new therapeutics for patients suffering from vancomycin-resistant enterococci (VRE)-dominated intestinal microbiota who are more susceptible to nosocomial infections (57–61).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Enterococcus faecalis* was grown in brain heart infusion broth (BHI) and cultured at 37°C under static or shaking (200 rpm) conditions, as indicated below. Preparation of inocula for biofilm and planktonic assays was performed as previously described (26). The bacterial strains used are listed in Table S2. Where appropriate, strains harboring pMSP3535 plasmids were selected using 100 µg/ml erythromycin (Sigma-Aldrich, USA), and induction of gene expression was performed using 5 µg/ml nisin from *Lactococcus lactis* (Sigma-Aldrich, USA). BHI was supplied by Becton, Dickinson and Company, Franklin Lakes, NJ. TSB and agar were supplied by Oxoid, Inc., Ontario, Canada. Metals were filtered, sterilized, and supplemented during medium preparation in autoclaved TSBG medium. For experiments using ferric chloride only, metal was supplemented in TSBG medium, and they were autoclaved together. Magnesium chloride, anhydrous (≥98%), copper chloride dihydrate (≥99%), ferric chloride, anhydrous (≥99%), and heme (≥90%) were supplied by Sigma-Aldrich, St. Louis, MO, USA. Manganese chloride tetrahydrate and zinc chloride were supplied by Merck Millipore, Singapore.

Protein homology determination. The *E. faecalis* OG1RF MntE (GenBank accession number AEA93276.1) amino acid sequence (389 amino acids) was queried against the nonredundant GenBank coding sequence (CDS), including *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Escherichia coli* taxonomy using the NCBI blastp online tool.

General cloning techniques. The nucleotide sequence of *mntE* was obtained from the *E. faecalis* OG1RF genome via BioCyc (62). The Wizard genomic DNA purification kit (Promega Corp., Madison, WI) was used for isolation of bacterial genomic DNA (gDNA), and the Monarch plasmid miniprep kit (New England BioLabs, Ipswich, MA) was used for purification of plasmids for gene expression and construction of the complement mutant. The Monarch DNA gel extraction kit (New England BioLabs, Ipswich, MA) was used to isolate PCR products during PCR. The In-Fusion HD cloning kit (TaKaRa Bio USA, Inc.) was used for fast, directional cloning of DNA fragments into the expression vector. All plasmids used in the study are listed in Table S2. T4 DNA ligase and restriction endonucleases were purchased from New England BioLabs (Ipswich, MA). Colony PCR was performed using Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and PCR of genes of interest for plasmid construction was performed using Phusion

DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Ligations were transformed into *E. coli* Dh5 α cells. Plasmids derived in this study were confirmed by sequencing of purified plasmids.

Strain construction. To construct the *mntE* complementation plasmid, primers (*mntE*_F' and *mntE*_R'; Table S3) were designed with BamHI and SmaI restriction sites flanking the gene of interest to generate DNA fragments as templates. In-Fusion cloning (TaKaRa Bio USA, Inc.) was performed using primers (*mntE*_F'_Infusion and *mntE*_R'_Infusion) with at least 15 bp complementary sequence for ligation into the nisin-inducible vector pMSP3535, which was also digested with the same restriction enzymes. The pMSP3535::*mntE* plasmid was generated in *E. coli* Dh5 α , verified by sequencing, and transformed into *E. faecalis* as described previously (37).

Biofilm assay. Bacterial cultures were normalized as previously described (26), inoculated in TSBG in a 96-well flat-bottom transparent microtiter plate (Thermo Scientific, Waltham, MA, USA), and incubated at 37°C under static conditions for 5 days unless specified otherwise. Strains harboring pMSP3535 complementation plasmid were grown in the presence of erythromycin. Adherent biofilm biomass was stained using 0.1% wt/vol crystal violet (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 30 min. The microtiter plate was washed twice with phosphate-buffered saline (PBS) followed by crystal violet solubilization with ethanol:acetone (4:1) for 45 min at room temperature. Quantification of adherent biofilm biomass was measured by absorbance at an optical density at 595 nm (OD₅₉₅) using a Tecan Infinite 200 PRO spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

Plate-assisted planktonic growth assay. Bacterial cultures were normalized as previously described (26) and further diluted by a dilution factor of 200. Diluted cultures were then inoculated into fresh medium at a ratio of 1:25, which is 8 μ l of the inoculum in 200 μ l of medium, and incubated at 37°C for 18 h, and the absorbance at OD₆₀₀ was measured using a Tecan Infinite 200 PRO spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland) at 15-min intervals (with shaking prior to each measurement).

Planktonic growth kinetic assay. Bacterial cultures were normalized as previously described (26) and inoculated into fresh medium at a ratio of 1:1,000 in 30 ml of medium in 50-ml conical tubes. The tubes were incubated with shaking for 8 h at 200 rpm at 37°C. At indicated time intervals, 100 μ l and 1 ml of culture were removed for CFU enumeration and optical density measurement, respectively.

Macrocolony assay. Bacterial cultures were normalized as previously described (63) and spotted onto a TSBG agar plate at 5 μ l per spot. Agar plates were supplemented with metals where appropriate and incubated for 120 h at 37°C. Macrocolonies were excised, vortexed, and resuspended in 3 ml PBS and were serially diluted for CFU enumeration. Then, 5 μ l of dilution from each well was spotted onto the agar plates and incubated at 37°C overnight for subsequent calculation of CFU/ml.

Inductively coupled plasma mass spectrometry. Biofilms were cultured under static conditions at 37°C for 24 h. After incubation, biofilms were scraped from the surface, resuspended in 1 ml PBS, and normalized to an OD of 1. Duplicate samples of OD 1 were plated to determine the CFU/ml, and subsequent metal quantification measurements were normalized against the CFU/ml of the sample. OD 1 normalized biofilm cells were pelleted at 14,000 rpm for 2 min, and the supernatant was discarded. Preparation of cell pellets for inductively coupled plasma mass spectrometry (ICP-MS) was performed as previously described (26) with minor modifications. Cell pellets were suspended in 300 μ l of lysozyme from chicken egg white (20 mg/ml) (Sigma-Aldrich, USA) (20 mg/ml) for 30 min at 37°C, washed with 1 ml PBS, and pelleted. At a ratio of 2:1, 70% nitric acid (Sigma-Aldrich, USA) and 30% hydrogen peroxide (Sigma-Aldrich, USA) were added to the normalized lysozyme-treated biofilm cells and incubated at room temperature for 3 days to allow complete digestion. The digested samples were diluted with 3.4 ml liquid chromatography-mass spectrometry (LC-MS)-grade water and filtered using a 0.2- μ m membrane prior to analysis using ICP-MS. Analysis of trace metals in samples was performed using ICP-MS model Elan-DRCE, Meinhard nebulizer model TR-30-C3 (Perkin Elmer; model N8122006 [Elan Standard Torch]). During each ICP-MS run, separate samples of blanks (digestion solution without cells) were run to ensure little or no background metal interference. Three biological samples were run in each experiment, and a minimum of three nonconsecutive experiments were performed. The data obtained and shown were the average of three experiments.

Quantitative real-time PCR and RNA sequencing. Biofilms were grown in a 6-well plate for 24 h at 37°C under static conditions. Postincubation, spent medium was removed and biofilms were suspended in PBS prior to being dislodged using a cell scraper. Biofilm cultures were centrifuged at 14,000 rpm for 2 min at room temperature to remove supernatant. Biofilm cell pellet was incubated with lysozyme from chicken egg white (10 mg/ml) (Sigma-Aldrich, USA) for 30 min at 37°C and was centrifuged at 14,000 rpm for 2 min at room temperature to remove supernatant prior to cell lysis. RNA extraction was performed in a Purifier filtered PCR enclosure using the PureLink RNA minikit (Invitrogen, USA) according to the manufacturer's instructions. RNA purification and removal of DNA were performed using a Turbo DNA-free kit (Thermo Fisher, USA) and an Agencourt RNAClean XP kit (Beckman Coulter, USA). Measurement of RNA yield and quality were performed using a Qubit RNA HS assay kit (Thermo Fisher, USA) and an RNA ScreenTape system and 2200 TapeStation (Agilent, USA). Synthesis of cDNA was performed using a SuperScript III first-strand system (Invitrogen, USA). Quantitative real-time PRC (qRT-PCR) using cDNA was performed using a Kapa SYBR fast qPCR master mix kit (Sigma-Aldrich, USA) and an Applied Biosystems StepOne Plus real-time PCR system. The expression of *ebpC*, *ebpR*, *mntE*, and *gyrA* was measured using the primer pairs listed in Table S3. For each primer set, a standard curve was established using genomic DNA from *E. faecalis* OG1RF. Normalized amounts of cDNA were used to determine the relative fold change in gene expression compared to *E. faecalis* OG1RF biofilm grown in TSBG. For RNA sequencing, rRNA depletion was performed after RNA purification using a Ribo-Zero rRNA removal kit (Illumina, USA). cDNA library synthesis was performed using a NEBNext RNA first-strand

system and a NEBNext Ultra directional RNA second-strand synthesis module (New England BioLabs, USA). Transcriptome library preparation was performed using 300-bp paired-end Illumina sequencing. RNAseq raw data files have been submitted to the NCBI SRA database (SRA accession number PRJNA610696; release date, 30 April 2021).

Mouse gastrointestinal tract colonization model. Six-week old male C57BL/6NTac mice were administered ampicillin (VWR, USA) in their drinking water (1 g/liter) for 5 days as previously described (58, 64). Mice were then given 1 day of recovery from antibiotic treatment prior to administration of approximately $1 \text{ to } 5 \times 10^8$ CFU/ml *E. faecalis* ($\text{OD}_{600\text{nm}}$, 0.5) in the drinking water for 3 days as previously described (65). Before and after infection, mice were monitored for signs of disease and weight loss. All animal experiments were approved and performed in compliance with the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC), under protocol ARF-SBS/NIE-A0280. At the indicated time points, the small intestine, colon, and cecum were harvested. Tissue samples were homogenized in PBS, serially diluted in PBS, and spot-plated on BHI agar with 10 mg/liter colistin, 10 mg/liter nalidixic acid, 100 mg/liter rifampin, and 25 mg/liter fusidic acid for CFU enumeration. All antibiotics were obtained from Sigma-Aldrich, USA.

Statistical analyses. Data from multiple experiments were pooled, and appropriate statistical tests applied, as indicated in the figure legends. Statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA). An adjusted *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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L.N.L. and K.A.K. conceived of and designed the study, analyzed the data, and wrote the manuscript. L.N.L. performed all of the experiments. J.J.W. assisted with the animal experiments, and K.K.L.C. analyzed the RNA sequencing data. All authors edited and approved the final manuscript.

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