AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews®

REVIEW

Adaptation to Adversity: the Intermingling of Stress Tolerance and Pathogenesis in Enterococci

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Citation Gaca AO, Lemos JA. 2019. Adaptation to adversity: the intermingling of stress tolerance and pathogenesis in enterococci. Microbiol Mol Biol Rev 83:e00008-19. https://doi.org/10.1128/MMBR.00008-19.

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Published 17 July 2019

SUMMARY *Enterococcus* is a diverse and rugged genus colonizing the gastrointestinal tract of humans and numerous hosts across the animal kingdom. Enterococci are also a leading cause of multidrug-resistant hospital-acquired infections. In each of these settings, enterococci must contend with changing biophysical landscapes and innate immune responses in order to successfully colonize and transit between hosts. Therefore, it appears that the intrinsic durability that evolved to make enterococci optimally competitive in the host gastrointestinal tract also ideally positioned them to persist in hospitals, despite disinfection protocols, and acquire new antibiotic resistances from other microbes. Here, we discuss the molecular mechanisms and regulation employed by enterococci to tolerate diverse stressors and highlight the role of stress tolerance in the biology of this medically relevant genus.

KEYWORDS *Enterococcus*, acid stress, antibiotic resistance, nutrient stress, opportunistic infections, oxidative stress, stress response

INTRODUCTION

The gastrointestinal tract (GI) tract is a dynamic environment subject to substantial fluctuations in nutrient availability and content, oxygen tension, and pH. Bacteria transiting as well as colonizing the gut must therefore be equipped with mechanisms to adapt and persist in this dynamic environment. The pH of the human GI tract can vary by as much as 6 orders of magnitude from the highly acidic environment of the lower stomach to the mildly alkaline environment in the distal small intestine. Oxygen content and redox potential also drop dramatically from the oxygenated environment of the stomach to the strongly reducing environment of the colon lumen. Mucosal surfaces adjacent to the epithelium of the large intestine, however, may remain microaerobic by diffusion of oxygen from capillaries within the epithelial layer (1). Furthermore, gut bacteria have to contend with a changing nutritional landscape due to variability in host diet and transit along the GI tract as organisms move along with the food bolus propelled by peristaltic force. The gut microbiota must also cope with the production of numerous host-derived molecules, including bile, digestive enzymes, and antimicrobial peptides such as defensins and cathelicidins, which can be inhibitory or even lethal (2, 3). In perhaps the most extreme case, when GI commensals are excreted into the environment, they must endure rapid and dramatic changes in many physiochemical conditions simultaneously.

The genus *Enterococcus* is a group of low-GC content Gram-positive cocci that currently consists of approximately 50 species (4). To date, most research efforts have been focused on *Enterococcus faecalis* and *Enterococcus faecium* due to their long-standing association with human disease and emergence as leading causes of drug-resistant hospital-acquired infections. Although *E. faecalis* accounts for the majority of enterococcal infections, the number of nosocomial *E. faecium* infections is increasing and is attributable to the high incidence of multidrug resistance (MDR) in this species (5). In addition to the capacity to develop MDR, the enterococci are well adapted to survive the environmental extremes needed to successfully transit through the GI tract, into the environment, and back again. Enterococci are also found in other human body sites such as the skin, oral cavity, and female urogenital tract. Oral enterococci may seed and influence the colonic population, and populations on the skin and in the reproductive tract likely originate from the colonic population, but these relationships have not been firmly established. The ability to inhabit diverse human body sites likely reflects many of the properties that enable enterococci to colonize a broad range of

hosts from insects to humans (4). Enterococci also occur as contaminants of food and in the microbiota of soil, water, and vegetation (4). As enterococci are extensively auxotrophic, it is unlikely that they actively divide in all of these ecologies, but they clearly persist.

Durability and metabolic flexibility in diverse environments typify the genus *Enterococcus*. The enterococci are able to utilize a diverse set of carbohydrates to support growth (6, 7). In addition, members of the genus can grow in temperatures ranging from 8 to 45°C, broad pH values up to 9.6 and as low as 4.8, 6.5% NaCl, and 40% bile salts (8, 9). In fact, the ability to withstand harsh chemical and environmental stresses lethal to most non-spore-forming bacteria was initially used to separate enterococci from the morphologically and metabolically similar streptococci. This remarkable stress resilience is enhanced following exposure to sublethal stress conditions. For example, exposure to a sublethal temperature of 50°C for a period of 30 min increased *E. faecalis* ATCC 19433 resistance to lethal challenge at 60°C by 6 logs (10). Cross-tolerance has also been observed in which adaptation to a hyperosmotic environment (6.5% NaCl) led to enhanced survival when enterococci were exposed to lethal stresses, such as detergents (0.3% bile and 0.017% SDS), 22% ethanol, 45 mM hydrogen peroxide (H_2O_2), and heat (62°C) (11). Similarly, adaptation to pH 10.5 enhanced survival by more than 3 logs in 0.3% bile and, to a lesser extent, H_2O_2 and 62°C (12). Collectively, these factors situate *Enterococcus* as a model genus to study stress tolerance in commensal and pathogenic bacteria.

E. faecium and *E. faecalis* and other species of *Enterococcus* are commensal members of the gut microbiota although the enterococci typically comprise a minority of the total gut population (13, 14). Enterococcal pathogenesis has been recognized for over a century, beginning with the isolation of a Gram-positive coccus, described as "very hardy and tenacious to life," from a patient with acute infective endocarditis (IE) (15). This strain, initially named *Micrococcus zymogens* for its proteolytic and hemolytic properties and later renamed *Streptococcus zymogens*, is now recognized to be *E. faecalis* (15, 16). For much of the 20th century, enterococci were sporadically isolated from cases of endocarditis, septicemia, urinary tract infections, and wound infections. These infections were often polymicrobial, leading to ambiguity if enterococci were the causative agents or hitchhikers, earning them a reputation as microbes of limited pathogenicity. It was not until the emergence of MDR strains, endemic to hospital wards and responsible for sustained cases of bacteremia and other diseases (17), that the existence of more virulent hospital lineages became apparent. This was quickly compounded by the appearance of isolates of vancomycin-resistant enterococci (VRE) from European hospitals in the late 1980s and shortly thereafter in the United States. (18). At this point, enterococci became broadly appreciated as pathogens highly refractory to conventional chemotherapeutics (19). Despite the recent introduction of new antibiotics, such as daptomycin and linezolid, *E. faecalis* and *E. faecium* remain leading causes of MDR infections (20, 21).

In comparison to other Gram-positive pathogens, like *Staphylococcus aureus* or *Streptococcus pyogenes*, enterococci do not produce a large number of toxins or other mediators of inflammation and tissue damage (5). On the other hand, *E. faecalis* employs a multitude of strategies to subvert the host immune responses. For a thorough appreciation of those strategies, we refer the reader to a recent review that highlights the mechanisms used by *E. faecalis* to suppress, evade, or inactivate host immune responses (22). Most of the factors that lead to enterococcal virulence in animal models are associated with tissue colonization, such as enterococcal surface protein (Esp) (23), endocarditis- and biofilm-associated pili (Ebp) (24), and the collagen adhesins of *E. faecalis* (Ace) (25–27) and *E. faecium* (Acm) (27, 28), with the exception of the acute cytotoxicity conferred by cytolysin (29). No single virulence factor or set of factors appears to be an absolute requirement for its pathogenicity (30). The acquisition of antibiotic resistance traits, mainly through horizontal gene transfer, has provided enterococci with a strong advantage for survival in the hospital setting, while the characteristic resilience of this genus enhances the infectivity of the resultant MDR

strains. To successfully spread from an infected individual to a susceptible host, enterococci must survive the forbidding trip on communal surfaces, often treated with disinfectants, and reestablish colonization within the new host in spite of host innate immune defenses and preexisting microbial competitors. For *E. faecalis* and *E. faecium*, stress tolerance and pathogenesis are intimately linked. This same intrinsic durability is also a key transmission factor for movement of commensal enterococci between hosts and for the persistence of enterococci in the environment. In the present review, we describe the mechanisms by which the enterococci respond to and survive diverse stressors and highlight the role of stress tolerance in the biology of this medically and environmentally important genus.

PIONEERING STUDIES

Until the 1990s, little research had been conducted to decipher the mechanisms behind the impressive multistress tolerance of enterococci. A series of pioneering studies conducted by a group at the University of Caen (France) employed two-dimensional gel electrophoresis to examine changes in the proteome of *E. faecalis* ATCC 19433 subjected to a variety of environmental and chemical stresses. By focusing on proteins with enhanced expression under stress, the investigators aimed to identify candidate proteins and pathways critical for responding to environmental change. The first of these studies compared stress responses between exposure to sublethal challenge with bile salts, low pH (pH 4.8), and heat (50°C) (31). In response to treatment with these three stressors, over 30 distinguishable proteins were induced in ^{35}S-labeled protein extracts (31). Nine induced proteins were expressed in all stress treatments, leading the authors to classify them as general stress proteins (31). These general stress proteins were postulated to provide nonspecific protection under conditions generally not permissive to growth (32). Despite the technical limitations of the time, two of the nine proteins were positively identified as the heat shock-inducible chaperones DnaK and GroEL (31). In addition to bile, acid shock, and heat, the DnaK and GroEL chaperones were also induced following the adaptation of *E. faecalis* to alkaline (pH 10.5) conditions (10) and treatment with ethanol (33). Over the following years, complementary proteome analyses were performed under osmotic stress (11), cold shock (34), and nutrient limitation (35–37). Glucose exhaustion and oligotrophic stress caused by dilution into tap water led to significant alterations in protein metabolism and a larger number of proteins induced than by other chemical and environmental stresses examined (37). Comparison of proteins commonly induced between these two nutritional stresses identified a strong overlap of 16 proteins, indicating that glucose starvation and oligotrophy trigger similar stress responses (37). Thus, a core set of proteins coordinates overlapping, yet distinct, stress responses of *E. faecalis*.

With the release of the first *E. faecalis* complete genome in 2003 (38) and the development of new tools for genetic manipulation, the field of enterococcal biology underwent an unprecedented transformation. In the subsequent decade, functional genomic, transcriptomic, and proteomic approaches provided a much more detailed analysis of the stress response mechanisms of enterococci. In the next sections, the contributions of specific genes and pathways to enterococcal stress tolerance, mainly that of *E. faecalis*, are discussed.

MOLECULAR MECHANISM AND REGULATION OF STRESS TOLERANCE

Oxidative Stress

The prokaryotic world emerged and evolved over many millennia in an anoxic environment (39). During these formative years, the enzymatic mechanisms and biochemical redox reactions underlying central metabolic pathways evolved without pressure to avoid or mitigate toxic interactions with molecular oxygen and the subsequent generation of reactive oxygen species (ROS). The emergence of cyanobacterial photosynthesis and the great oxidation event selected for bacteria with enzymatic mechanisms less susceptible to oxidation and with mechanisms to scavenge toxic ROS and repair oxidative damage to cellular components (40). Solvent-exposed iron-sulfur

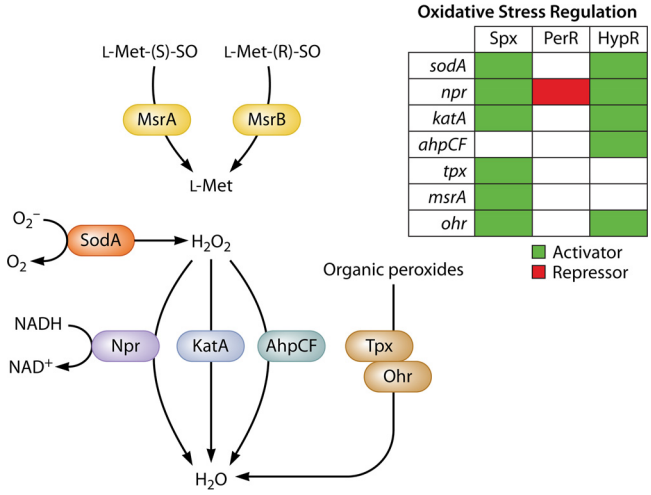

FIG 1 The management and regulation of redox stress. To combat both endogenous and exogenous sources of peroxide stress, enterococci contain three conserved peroxidase enzymes, Npr, AhpCF, and Tpx. Although all three enzymes enhance peroxide tolerance, Tpx activity is primarily restricted to mitigating the effects of organic peroxides. Catalase (KatA) is used to neutralize high concentrations of exogenous H_2O_2 and appears to act complementarily to Npr, which scavenges low-level endogenous H_2O_2 production. The manganese-dependent superoxide dismutase (MnSOD) reduces highly reactive superoxide to H_2O_2 that can be further reduced by the activity of peroxidases and catalase. (The presence of *katA* appears primarily restricted to *E. faecalis*.) If oxidants are not scavenged quickly enough and damage occurs to critical cellular proteins, the combined action of methionine reductases (MsrA and MsrB) reverses the oxidation of methionine thiols to restore protein structure and function. The transcriptional control of these antioxidant mechanisms is carried out by at least three regulators: Spx, PerR, and HypR. Spx and HypR are activators of antioxidant genes, while PerR functions as a transcriptional repressor in the absence of peroxide stress.

(Fe-S) clusters appear to be the main targets of ROS, particularly H_2O_2, leading to generation of highly reactive superoxide anion (O_2^-) and hydroxyl radical (HO^-). These ROS are able not only to directly damage DNA, proteins, and membrane lipids but also to increase the release of iron from Fe-S clusters that bind macromolecules, accelerating ROS toxicity (40). Thus, the response to ROS stress typically targets Fe-S cluster protection and repair, regulation of cellular iron, DNA and protein repair, ROS scavenging, and ROS degradation.

Detoxifying ROS is a central challenge for enterococci which endogenously produce ROS, including H_2O_2 and O_2^- (41, 42). Hydrogen peroxide is formed as a by-product of the aerobic fermentation of glycerol 3-phosphate to dihydroxyacetone phosphate by α-glycerophosphate oxidase, and *E. faecalis* is capable of secreting millimolar amounts of H_2O_2 into the extracellular milieu when grown aerobically in the presence of glycerol (43, 44). The production of superoxide (O_2^-) appears to be species or even strain specific and not a trait conserved among enterococci (45). Superoxide production occurs when enterococci are grown in the absence of heme and results from univalent oxidation of demethylmenaquinone (DMK), producing a semiquinone radical that can react with oxygen to produce O_2^- (41). To balance production of endogenous ROS, enterococci must also possess strong antioxidant capabilities and a redox-sensing regulatory network controlling antioxidant pathways (Fig. 1). Evidence of such a response comes from the 200-fold increase in survival upon H_2O_2 challenge following adaptation of *E. faecalis* ATCC 19433 to subinhibitory concentrations of H_2O_2 (46). In addition, growing evidence indicates that these potent antioxidant defenses also protect enterococci during infection from host-derived sources of ROS, including monocytes and neutrophils (47–50).

Molecular mechanisms of oxidative stress. (i) Catalase. The main function of catalase is to catalyze the dismutation of H_2O_2 to water and dioxygen (Fig. 1). Although enterococci are catalase negative when grown on most laboratory media, long before genome sequencing, it was noted that some species displayed catalase activity under

certain conditions (51). The catalase activity of *E. faecalis* was first described by Clarke and Knowles but only when cells were grown aerobically and supplemented with hematin (51). The expression of catalase and subsequent incorporation of heme into the apo-catalase are oxygen dependent, explaining why catalase activity is dependent on both heme and aerobic culture conditions (52, 53). While catalase activity had been known for many years, the *katA* gene encoding the *E. faecalis* cytoplasmic catalase was not characterized at the molecular level until 2002 (54). Among the enterococcal genera, the *katA* gene appears to be primarily restricted to *E. faecalis* and certain environmental isolates such as *Enterococcus haemoperoxidus*, *Enterococcus saccharolyticus*, and *Enterococcus sulfureus* (Fig. 2; see also Table S1 in the supplemental material). A *katA*-deficient mutant of *E. faecalis* OG1RF showed a modest reduction in survival when treated with H_2O_2 and could not be rescued to the level of the wild-type strain by addition of hemin (55). Interestingly, the *katA* mutant phenocopied the wild-type when grown aerobically in the presence of glycerol, indicating that catalase is more important for resistance to exogenous rather than endogenously produced H_2O_2 (55). The importance of catalase during the course of mammalian infection remains to be determined but could prove significant considering that neutrophils and monocytes are important barriers to enterococcal infection and exert part of their bactericidal activity through an oxidative burst, which includes H_2O_2 (56–58).

(ii) Peroxidases. In many organisms, detoxification of peroxides is a function of catalase. As mentioned above, only a subset of species within the *Enterococcus* genus produce a heme-dependent catalase (KatA) (51). Thus, to mitigate the damaging effects of peroxides in diverse environments, the enterococci employ multiple peroxidases (Fig. 1).

NADH peroxidase (Npr) is a unique flavin-based enzyme capable of reducing H_2O_2 to water. The Npr of *E. faecalis* has been characterized both structurally and functionally (59). The low K_m of Npr for H_2O_2 provides a potential advantage over catalase, allowing Npr to scavenge small amounts of H_2O_2 when catalase is poorly active (51, 60). The most likely biological function of Npr is to degrade endogenous H_2O_2 produced from α-glycerophosphate oxidase, but the permeability of bacterial membranes to H_2O_2 suggests that Npr may also protect against exogenous H_2O_2 (61). Consistent with this idea, deletion of *npr* resulted in significant impairment of *E. faecalis* JH2-2 to withstand oxidative challenge (50). Compared to growth of the wild type, a Δ*npr* strain was unable to grow aerobically on glycerol, showed increased H_2O_2 production, and had a drastic reduction in survival following lethal challenge with H_2O_2 (50). Surprisingly, the Δ*npr* strain showed no significant reduction in intracellular survival in murine macrophages or attenuated virulence in intraperitoneally infected mice (50), indicating that additional strategies of peroxide resistance are used by *E. faecalis* to survive in these H_2O_2-rich host environments.

Thiol peroxidases are an expansive class of enzymes that can reduce a broad-spectrum of hydroperoxides. The known substrates of thiol peroxidases include the following: H_2O_2; primary, secondary, and tertiary organic hydroperoxides; and peroxynitrite (62). In contrast to NADH peroxidase, thiol peroxidases catalyze the reduction of peroxides without the use of any cofactors or prosthetic groups. Instead, peroxides are degraded by oxidizing two active-site cysteines to form a disulfide bridge. The disulfide bond is then reduced by enzymes containing either glutathione, thioredoxin, or, in some cases, dithiols like lipoate as a reductant (62). The genome of *E. faecalis* is known to encode three thiol peroxidases: the alkyl hydroperoxidase (*ahpCF*) complex, organic hydroperoxidase resistance (*ohr*) protein, and a thiol peroxidase (*tpx*) (50, 63). Of the AhpCF complex, *ahpC* encodes the thiol-specific peroxidase while *ahpF* encodes an NADH-dependent thioredoxin-disulfide reductase responsible for reducing the intermolecular disulfide bonds of AhpC to return the enzyme to its active state (64). The Tpx of *E. faecalis* is structurally most similar to atypical 2-cysteine peroxiredoxins where both cysteines involved in disulfide bond formation are located on the N-terminal and C-terminal portions of the protein. This allows Tpx to form an intramolecular disulfide bond to reduce peroxides rather than through intermolecular disulfide bonds in

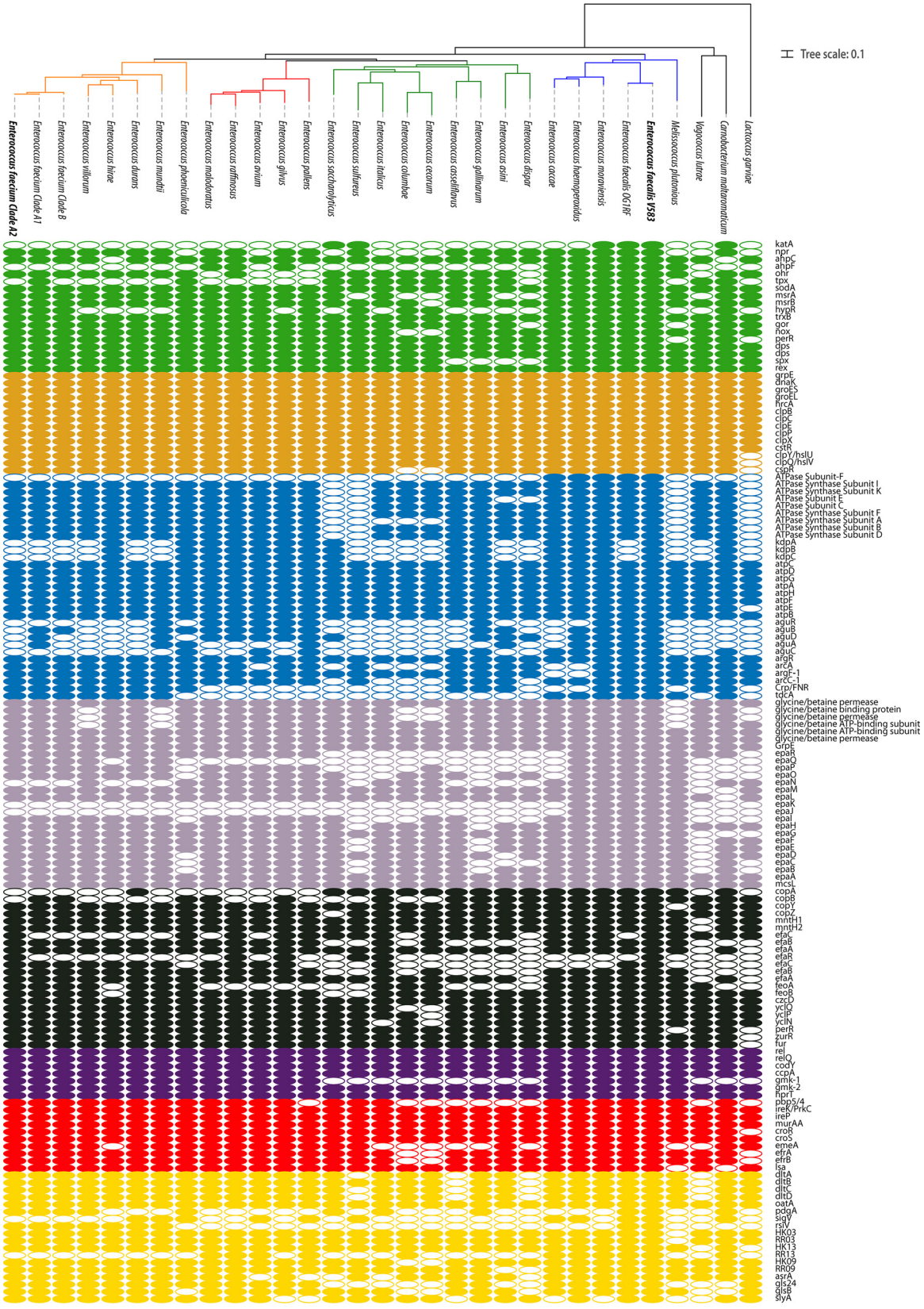

FIG 2 Distribution of stress resistance proteins across the *Enterococcus* genus. Orthologues of stress proteins described in this review, identified by their respective loci in *E. faecalis* V583, were mapped onto 28 diverse *Enterococcus* spp. and three closely related genera as outgroups. Orthologous proteins are grouped by the corresponding stress condition to which the protein confers resistance. Enterococcal species are color coded by clade. Orthogroups and phylogenetic analysis are based on data available in Lebreton et al. (339).

adjacent homodimers characteristic of other thiol peroxidases (50, 62). Ohr is unique in that it requires the consumption of dithiols with strongly negative redox potentials, likely dihydrolipoic acid, for the reduction of its disulfide bonds and, as the name implies, exhibits a strong preference for organic peroxides over H_2O_2 (65).

The loss of any one of these peroxidases generally decreases tolerance of *E. faecalis* JH2-2 to oxidative stresses (50). However, the function of all three peroxidases is not redundant: when exposed to H_2O_2, both the Δ*ahpCF* and Δ*tpx* strains had over a 1-log reduction in survival (50), whereas the survival of the Δ*ohr* strain was impaired only by addition of the organic peroxide *t*-butyl hydroperoxide (63). Moreover, only strains lacking *tpx* showed impaired intracellular survival in murine macrophages and attenuated virulence in a mouse peritonitis model (50), suggesting that organic peroxides may be the most relevant stressors in both of these environments. Notably, the *Escherichia coli* Tpx was shown to preferentially target hydroperoxides and lipid peroxides (66, 67) that may result from the macrophage oxidative burst. A genomic comparison of the *E. faecalis* AhpCF, Ohr, and Tpx against sequenced members of the *Enterococcus* taxon shows that these three proteins are conserved and therefore likely contribute to peroxide tolerance across the genus (Fig. 2; Table S1).

(iii) MnSOD. Superoxide dismutase converts O_2^- into H_2O_2 and molecular oxygen (Fig. 1) and is considered the primary bacterial defense against superoxide (68, 69). Superoxide dismutase enzymes are classified based on their metal cofactor: iron, copper-zinc, or manganese. All aerobic or facultative aerobic bacteria contain at least one superoxide dismutase enzyme (69), and enterococci are no exception as their genomes encode *sodA*, a manganese-dependent superoxide dismutase (MnSOD) (70). In agreement with the proposed importance of MnSOD to oxidative stress tolerance, deletion of *sodA* in *E. faecalis* JH2-2 results in a reduced growth rate in the presence of sublethal concentrations of H_2O_2 and a several-log reduction in survival toward menadione, *t*-butyl hydroperoxide, and H_2O_2 (48). Superoxide is also produced as a byproduct of antimicrobial redox cycling agents (e.g., naphthoquinone, menadione, and paraquat), and the phagocyte NADPH oxidase (40, 71, 72). As would be expected based on the importance of superoxide generation to antimicrobial action of professional phagocytes, the *sodA* mutant was less able to survive intracellularly within murine macrophages (48).

(iv) MetSO. Although enterococci are equipped with many antioxidant pathways to target and remediate oxidants before they damage cellular machinery, protein damage may still occur. Methionine is among the most oxidation-sensitive amino acids. Upon oxidation, methionine forms methionine sulfoxide (MetSO), which is composed of two stereoisomers (73). If left unrepaired in a protein, these oxidized residues can lead to changes in conformation or hydrophobicity and, ultimately, loss of function (74). As shown in Fig. 1, reduction of MetSO is carried out by two methionine sulfoxide reductases (MsrA and MsrB) that are specific to only one of the isomeric forms of MetSO (75, 76). Studies of *msrA* and/or *msrB* deletion mutants in other bacterial species show mixed phenotypes when strains were subjected to exogenous oxidative stress, and a limited number of studies have addressed their respective contributions to bacterial virulence (77–81).

The genome of *E. faecalis* encodes one copy of the *msrA* and *msrB* genes at different chromosomal locations (49). Deletion of either *msrA* or *msrB* in *E. faecalis* results in delayed growth in the presence of high concentrations of H_2O_2 (49). Also, deletion of both *msrA* and *msrB* is additive, leading to an even greater reduction in growth rate than that of either single deletion mutant (49). Consistent with the observation that MsrAB contributes to oxidant tolerance at elevated H_2O_2 concentrations, the *msrA* and *msrB* deletion mutants were less able to survive within activated murine macrophages (49). When mice were infected either intraperitoneally or transurethrally, the Δ*msrA*, Δ*msrB*, and Δ*msrA* Δ*msrB* strains showed reduced bacterial loads in the liver and kidneys (49). Like SodA, Npr, and the thiol-based peroxidases, MsrA and MsrB are highly conserved across *Enterococcus* species (Fig. 2; Table S1).

Transcriptional regulation of the oxidative stress response. (i) HypR. The LysR family transcriptional regulator OxyR is primarily responsible for inducible H_2O_2 resistance in Gram-negative bacteria. LysR family regulators are autoregulated, employ a helix-turn-helix motif to bind DNA, and interact with 15-bp inverted repeats located upstream of the translational start site (82). Well-characterized members of the OxyR regulon that are important for peroxide resistance in *E. coli* include the iron binding protein Dps, glutathione reductase, glutaredoxin, catalase, alkyl hydroperoxidase, and the Fur transcriptional repressor of iron transport (83). Although OxyR homologs are absent from *Firmicutes* (84), a search for putative transcriptional regulators of the LysR family in *E. faecalis* V583 revealed the presence of five genes encoding proteins with some sequence similarity to the *E. coli* OxyR homolog. Mutation of only one of these five putative regulatory genes (*EF2958*) resulted in enhanced sensitivity to H_2O_2 exposure (85). In addition, this regulatory locus contributed specifically to H_2O_2 resistance. Survival was not altered in the presence of other oxidative agents, including hydroperoxides or the redox cycling oxidant menadione. Due to this specificity, EF2958 was named HypR (hydrogen peroxide regulator) (85). Transcriptional analysis revealed that HypR was responsible for regulation of the *ahpCF*, glutathione reductase (*gor*), *katA,* NADH oxidase (*nox*), *npr*, *ohr*, *sodA*, and thioredoxin reductase (*trxB*) genes during H_2O_2 stress (86). Survival of the *hypR* mutant in murine peritoneal macrophages was severely reduced compared to that of wild-type *E. faecalis*, a phenotype that may be attributable to the lack of *ahpCF* and *sodA* activation (48, 50).

(ii) PerR. PerR is an oxidant-sensing transcriptional regulator in many Gram-positive bacteria that, like OxyR in Gram-negative organisms, may have evolved from a common Fur-like iron binding regulator (87). In the Gram-positive model organism *Bacillus subtilis*, PerR is a key regulator of the adaptive response to peroxide stress (88, 89). Transcriptional repression by PerR requires coordination of a regulatory metal ion, either Fe^{2+} or Mn^{2+}, to bind DNA (90). In the iron-bound form, PerR is highly sensitive to oxidative stress. Interaction with peroxides leads to Fe-catalyzed protein oxidation (90), loss of bound Fe, and subsequent operon activation of antioxidant genes, including *katA*, *ohr*, *ahpCF*, and *mrgA*, encoding a homolog of the iron binding protein Dps (91). In the manganese-bound form, PerR exerts transcriptional repression and is virtually insensitive to H_2O_2 (92). Surprisingly, quantitative real-time PCR (qRT-PCR) studies in *E. faecalis* JH2-2 examining the expression level of eight genes with known or putative roles in oxidative stress revealed that only *npr* and *nox,* which encodes an intracellular oxygen-consuming NADH oxidase, showed signs of PerR-dependent transcriptional regulation (93). Despite the small regulatory footprint of PerR, a *perR* mutant was approximately 2-fold more resistant to H_2O_2 challenge, suggesting that PerR transcriptionally regulates important antioxidant genes involved in peroxide defense (93). A more thorough examination of the *perR* regulon (e.g., global transcriptional analysis) may offer new clues as to the constituent genes mediating this H_2O_2 tolerance.

(iii) Spx. Spx is an oxidative stress transcriptional regulator that is highly conserved in low-GC-content Gram-positive bacteria. Spx regulates transcription at the level of RNA polymerase (RNAP) by binding the C-terminal domain of the α-subunit to influence gene expression (94). Spx is a small (~15 kDa) regulatory protein containing a redox-sensing CXXC motif that modulates its activity (88). Under oxidizing conditions, the formation of a disulfide bond between the two cysteines results in a conformational change that facilitates Spx-RNAP interactions. Constituents of the Spx regulon in *B. subtilis* primarily include genes that function in thiol homeostasis, cysteine biosynthesis, and ROS detoxification (88). In contrast to the streptococci and lactococci, *E. faecalis* and the genomes of other sequenced enterococci encode only a single Spx protein. Transcriptional analysis reveals that Spx activates numerous detoxification genes, summarized in Fig. 1 (47). Surprisingly, Spx also represses the transcription of the oxidative stress regulator *perR* and the antioxidant genes *dps* and *ahpCF* (47). Both *ahpCF* and *dps* are known targets of PerR regulation, suggesting that control of *ahpCF* and *dps* by Spx may occur indirectly through *perR* (91, 93). Consistent with evidence

that Spx is critical for the activation of established antioxidant genes in *E. faecalis*, the Δ*spx* mutant exhibited poor growth under aeration or when exposed to exogenous oxidants (47). More importantly, intracellular survival of the Δ*spx* strain was strongly attenuated in murine macrophages and in a mouse model of foreign body-associated peritoneal infection (47). It seems that Spx is a major oxidative stress regulator and contributor to the pathophysiology of *E. faecalis* and, based on its high degree of conservation, other enterococcal species.

(iv) Rex. Bacterial growth requires a continuous rebalancing of the cellular redox potential including NAD^+ and NADH levels. Therefore, cellular metabolism must be adjusted in relation to oxygen content and nutrient availability in order to maintain appropriate NAD^+/NADH ratios. The Rex family of transcription factors respond to cellular NAD^+/NADH ratios and control the expression of genes that function in anaerobiosis, fermentative metabolism, and oxidative stress (95–97). To monitor NAD^+/NADH ratios, Rex has a dinucleotide binding pocket that, when occupied by NADH, leads to dissociation from DNA and activation of the gene subset under Rex control (98). Unlike the genomes of most bacteria, the genome of *E. faecalis* initially appeared to encode two putative Rex homologs exhibiting the required structural domains involved in dinucleotide and DNA binding. Deletion of one of these genes in strain OG1, *EF2638*, resulted in significant alterations in the cellular NAD^+/NADH ratio, and gel shift assays confirmed that the DNA binding capacity of EF2638 was NADH responsive (99). Growth of the *EF2638* (*rex*) mutant was negatively impacted by aeration and, particularly, by aerobic growth on glycerol (an experimental condition that produces H_2O_2) (99). Transcriptome analysis revealed that Rex represses genes primarily responsible for anaerobic metabolism and NADH consumption, including pyruvate formate lyase, fumarate reductase, auxiliary lactate dehydrogenase, and alcohol dehydrogenase (99). Surprisingly, genes directly involved in oxygen metabolism or ROS detoxification were not detected, suggesting that Rex contributes to antioxidant defense indirectly, by repressing ROS-generating pathways and by maintaining the NADH pools upon which peroxidases like Npr rely (Fig. 1) (99).

Summary and perspectives. Enterococci have been found in close proximity to the illeal and colonic epithelia that secrete ROS in response to commensal or pathogenic bacteria (100–102). It seems likely that the diverse pathways for sensing and detoxifying ROS ideally positioned enterococci to stably colonize the animal gut. Furthermore, the ability to detoxify endogenous and exogenous sources of oxidants enhances survival when enterococci transition from their gastrointestinal niche to the environment or encounter host phagocytes during the infectious process.

As mentioned before, of the two prominent human enterococcal pathogens, only *E. faecalis* possesses catalase activity, suggesting that *E. faecalis* may be inherently more resistant to exogenous H_2O_2 than *E. faecium*. In addition, we now know that enterococci possess at least four major transcriptional regulators (Spx, PerR, HypR, and Rex) responsible for their adaptive oxidative stress response (Fig. 1) and that these regulators contribute to oxidative stress tolerance through control of critical antioxidant genes and redox homeostasis. In future studies, it would be interesting to explore the possibility that an enhanced capacity to mitigate the deleterious effects of exogenous H_2O_2 contributes to the greater prevalence of *E. faecalis* in human infection, where ROS is constantly produced by the host immune response. Therefore, it is likely that a combination of numerous and sometimes exceptionally efficient antioxidant defenses (e.g., Npr and MnSOD), along with diverse and overlapping oxidant sensing regulators, contribute to the remarkable resistance of enterococci to oxidative stress.

Thermal Stresses

A defining characteristic of enterococci is their ability to grow in temperatures ranging from 10 to 45°C (8). Growth at the extremes of this range is important for the ability of enterococci to survive in the environment and in the GI tract of animals with high body temperatures, such as birds. In addition to growth in broad temperature ranges, enterococci can survive shorter exposure to temperatures as high as 62°C (33).

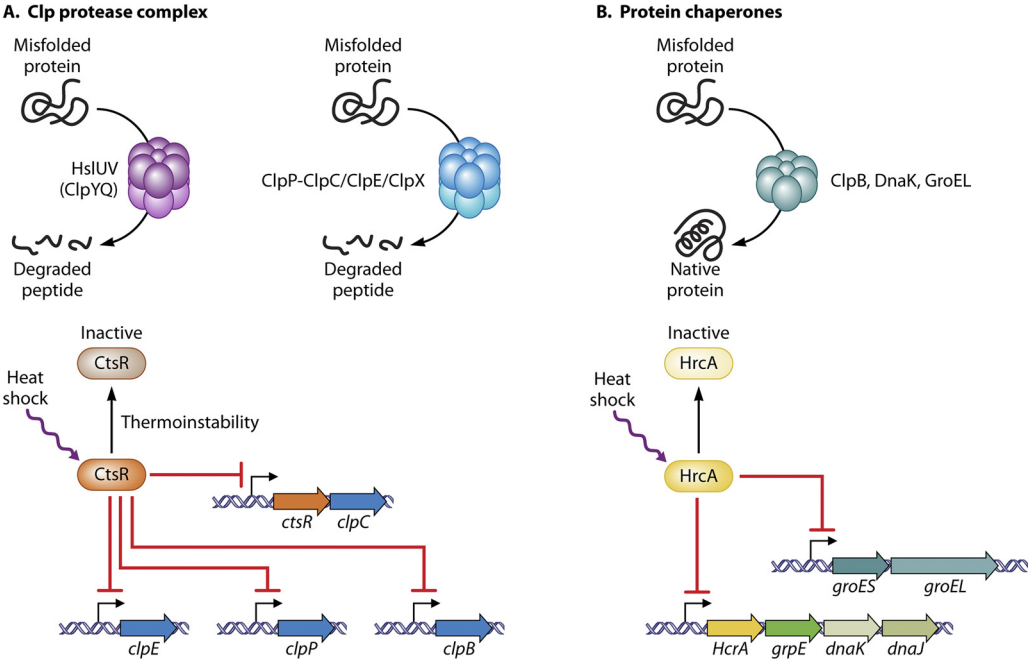

FIG 3 Coping with heat shock. (A and B) The protein-damaging effects of high heat stress in enterococci are managed by three molecular chaperones and at least one protease complex. The major chaperones GroEL and DnaK assist in refolding structurally disturbed proteins, returning them to their native conformation, or present irreversibly damaged proteins for degradation. GroEL and DnaK are repressed by HcrA, which becomes unstable under heat shock. The ClpP complex is a key multisubunit protease contributing to thermotolerance by degrading damaged and misfolded proteins. In enterococci, it consists of the ClpP peptidase paired with a partner Clp ATPase (ClpC, ClpE, or ClpX) that binds and transfers protein targets to ClpP for degradation. The noncanonical ClpB ATPase, which does not interact with the ClpP protease, also contributes to thermotolerance, likely through its chaperone activity. Transcription of the *clp* genes, except *clpX* and *clpB*, is repressed by CstR through what appears to be a thermostability mechanism homologous to HcrA. Enterococci also possess a second multisubunit protease, HslUV (also known as ClpYQ), but its contribution to thermotolerance or other stresses is not known.

Furthermore, exposure to mild heat induces a heat shock response, which dramatically increases the survival of *E. faecalis* at temperatures outside the growth-permissive range.

Heat stress. When mesophilic bacteria are exposed to high temperatures, they induce the synthesis of proteins called heat shock proteins, or HSPs. These HSPs fall into two classes: (i) molecular chaperones that assist in correct protein folding and assembly and (ii) ATP-dependent proteases that degrade permanently damaged proteins. The combined action of these highly conserved chaperones and proteases constitutes an essential protective mechanism that helps cells to avoid or cope with the appearance of misfolded or aggregated proteins during thermal stress (103).

The two major HSPs identified in the genus *Enterococcus*, and generally in all bacteria, are the molecular chaperones DnaK and GroEL. The genetic structure and transcriptional regulation of the *dnaK* and *groEL* operons have been examined in both *E. faecalis* and *E. faecium* (Fig. 3). In both species, these two operons are strongly induced at elevated temperatures (104, 105). Based on the presence of conserved CIRCE (controlling inverted repeat of chaperone expression) elements in their regulatory region and in the HrcA transcriptional repressor, both operons are classified as members of the class I heat shock regulon (Fig. 3).

Another important group of HSPs belongs to the class III heat shock regulon, regulated by the CtsR repressor. The class III heat shock regulon is composed of five genes encoding the ClpP peptidase and the Clp ATPases ClpC (cotranscribed with *ctsR*), ClpB, and ClpE. The ClpP peptidase associates with a Clp ATPase partner to form a functional and proteolytically active complex (Fig. 3). In addition to *clpB*, *clpC*, and *clpE*, the genome of *E. faecalis* encodes an additional ATPase not subject to CtsR control,

called ClpX (106). An equivalent complement of Clp ATPases also appears to exist across the enterococcal genus (Fig. 2; see also Table S1 in the supplemental material).

The ClpB protein is distinguished from the other three enterococcal Clp ATPases by the absence of a ClpP recognition tripeptide that allows interaction with ClpP. Rather than acting as part of the ClpP proteolytic complex, at least in *E. coli*, ClpB protein was shown to function as a molecular chaperone by interacting with DnaK to resolve protein aggregates (107). Although the mechanism of ClpB activity has not yet been characterized in an *Enterococcus* spp., the contributions of the *clpB* gene to cell physiology have been examined in *E. faecalis* OG1RF. The *clpB* gene was induced following heat shock, and this induction was repressed by CtsR (108). Interestingly, even in the absence of CtsR regulation, *clpB* still showed modest induction at 48°C, indicating that other factors may regulate the transcriptional activity of *clpB* (108). In *B. subtilis*, McsA and McsB proteins modulate proteolysis of CtsR through phosphorylation and subsequent activation of ClpC (109, 110). However, *E. faecalis* lacks the McsA and McsB modulators, suggesting that the DNA binding and transcriptional regulatory activities of CtsR may be instead controlled by its intrinsic thermal instability (111) and transcriptional autoregulation (112) (Fig. 3). The genus *Enterococcus* also possess an additional two-component protease composed of HslU and HslV (also known as ClpYQ), which resembles the 26S ubiquitin proteasome of eukaryotes. The ClpYQ protease contributes to the thermotolerance of *S. aureus* (113), but its relevance to the stress tolerance of enterococci remains to be determined.

Despite their nomenclature, HSPs are not specific to thermal stresses; these proteases and chaperones are known to more broadly contribute to bacterial stress tolerance and virulence. As noted in the early proteomic studies, the class I HSPs GroEL and DnaK accumulated under numerous stresses and were considered general stress proteins (12, 31). In *S. aureus*, a *dnaK*-deficient strain was more susceptible to oxidative stress and cell wall-active antibiotics and less virulent than its wild-type counterpart (114). Interestingly, DnaK and GroEL are essential in *Streptococcus mutans*, but knockdown of DnaK and GroEL levels significantly reduced acid and oxidative stress tolerance (115). Other than studies showing that the ClpB chaperone was important for the ability of *E. faecalis* to cause systemic infection in the invertebrate *Galleria mellonella* (108), the contributions of ClpP and the other Clp ATPases as well as of GroEL and DnaK to *E. faecalis* thermotolerance and other stress responses have not been directly assessed. Given the general importance of protein homeostasis to cellular physiology, it would not be surprising if HSP protease/chaperone systems are integrated into various stress responses in *E. faecalis* and other enterococci.

Cold stress. Temperatures lower than the permissive growth range impose several physiological changes in *Enterococcus* spp. These include the following: (i) reduction in membrane fluidity affecting functions like nutrient transport and protein secretion, (ii) increased stability of nucleic acid secondary structures reducing transcriptional and translational efficiency, (iii) slowed folding or misfolding of proteins, and (iv) reduced ribosome functionality (116). Numerous proteins are expressed to counter the effects of a temperature downshift, including fatty acid desaturases that increase membrane fluidity, RNA chaperones, transcriptional anti-terminators, low-temperature protein chaperones, and proteins that synthesize sugar cryoprotectants (116). These induced proteins have been further divided into two categories, cold shock proteins (CSPs) and cold acclimation proteins (CAPs). CSPs are induced in response to temperature downshift whereas CAPs are expressed during continuous growth in the low range of permissive temperatures.

E. faecalis is considered inherently resistant to cold shock as approximately 1% of cells survive up to six freeze-thaw (−20°C/37°C) cycles (9). The cold shock response is inducible in response to exposure to cold prior to lethal challenge at subfreezing temperatures. Preadaptation of *E. faecalis* for one doubling time at 10°C causes a 30% increase in survival per freeze-thaw cycle compared to survival of cells grown at 37°C, and additional adaptation time at colder temperatures further enhances this survival rate (9). Subsequent proteomic analysis of *E. faecalis* JH2-2 incubated at 8°C for one

generation (30 h) caused the induction of 11 CSPs. All of these CSPs were highly acidic (pKa $<$ 5.5) and small ($<$15 kDa in size) (34). Five of these CSPs were also induced during prolonged exposure to 8°C. In addition, five proteins were expressed only during long-term exposure, suggesting that there are distinct, yet overlapping, sets of proteins that contribute to cold shock and cold adaptation in *E. faecalis* JH2-2 (34). However, the identities of these CAPs and CSPs are yet to be determined.

Among the proteins that may be linked to cold shock or adaptive responses in the enterococci are RNA binding proteins. These proteins are necessary for the maintenance of proper RNA structure and gene expression under suboptimal temperatures (116). RNA binding proteins are also important for antimicrobial resistance in *S. aureus* and starvation survival in *B. subtilis* (117, 118). In *E. faecalis* EryS (an erythromycin-sensitive derivative of V583), a single cold tolerance gene has been investigated in some detail, i.e., the putative CspR cold shock RNA binding protein. *E. faecalis* CspR contributes to cell growth at lower temperatures, starvation survival, virulence in *G. mellonella*, and intracellular survival in macrophages (119). Future studies of the mechanism of *E. faecalis* CspR activity, in addition to the identification of the CSPs and CAPs, will surely advance our understanding of enterococcal thermal and nonthermal stresses in diverse habitats.

Summary and perspectives. The enterococci are notable among mesophilic bacteria for their ability to grow and survive under an unusually broad temperature range. The combination of transcriptional and proteomic studies strongly indicates that general stress proteins, such as the members of the Clp family and the class I HSPs GroEL and DnaK, are central to heat stress resistance in *E. faecalis* and perhaps in other enterococci (Fig. 3). There has been, however, little direct assessment of the contribution of these proteins to thermotolerance. We know even less about cold resistance in the enterococci, and the identities of CSPs and CAPs shown to accumulate during short-term and long-term cold stress remain elusive. Given the broader contributions of heat and cold shock regulons to general stress tolerance, these homeostatic mechanisms are likely to contribute more broadly to the rugged nature of enterococci. Thus, future studies of the cold stress response may reveal overlap in stress regulation and responses in the model organism *E. faecium* and, possibly, provide insight into the resilience of this species and of other members of the genus *Enterococcus*.

pH Stress

All bacteria must maintain an intercellular pH that is compatible with the function of enzymes necessary for cell growth. For most nonextremophiles, optimal intracellular pH varies relatively little (pH 7.5 to pH 8), while extracellular pH permissible for growth varies greatly in comparison (120). The small variance in cytoplasmic pH reflects the presence of underlying mechanisms that control cell permeability to protons and buffer production to counter either extreme in external pH. The ability to grow at a pH of 9.6 was initially used to separate *E. faecalis* from morphologically similar streptococci (8). Although not as well appreciated, the enterococci are also highly acid tolerant and are able to acidify batch culture medium to pH 3, an aciduricity comparable to that of the notoriously acid-tolerant dental pathogen *S. mutans* (121). The ability of the enterococci to grow and survive at a broad pH range has important implications in both human health and industrial and food processes. For example, the enterococci are prominent endodontic pathogens and a leading cause of root canal failure, due in part to their ability to survive alkalization of the root canal after calcium hydroxide treatment (122). Moreover, the survival of the enterococci under highly alkaline and acidic pH contributes to their importance in fermentation processes, particularly, the ripening of raw milk cheeses (123).

Alkaline stress. To maintain proper cytoplasmic pH under alkaline conditions, neutralophiles enhance the expression or activity of transport enzymes that promote proton uptake, metabolic pathways leading to the production of acidic buffers, and alteration of the cell envelope for better proton retention (124, 125). Of these mechanisms, proton/cation antiporters are key contributors to active pH homeostasis (Fig. 4).

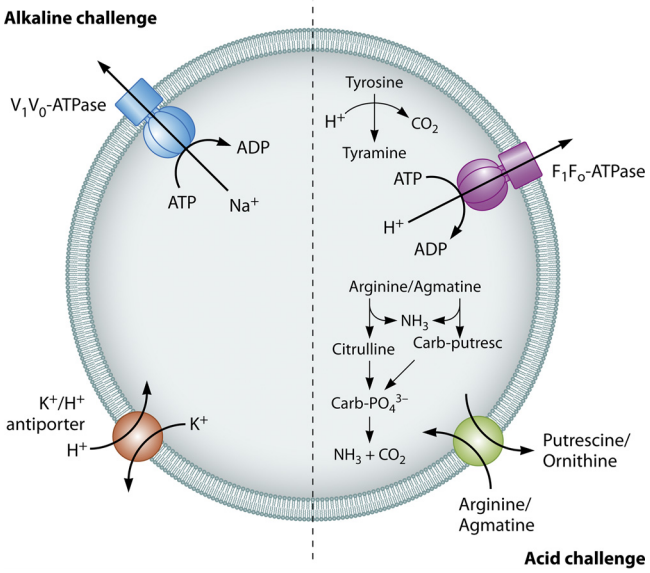

FIG 4 Maintenance of intracellular pH homeostasis during acid and alkaline challenge. Under alkaline conditions, *E. hirae* utilizes the V_1V_0-ATPase to pump sodium from the cell through ATP hydrolysis to create a strong electrochemical potential across the cell membrane. This electrochemical gradient aids in compensating for the unfavorable proton gradient (ΔpH) but also drives the import of protons through the potassium/proton antiporter to maintain a neutral intracellular pH. When exposed to acidic conditions, enterococci utilize active proton export strategies in conjunction with amino acid catabolism to buffer the cytoplasm. The enterococcal F_1F_o-ATPase functions in the hydrolytic direction to consume ATP and excrete protons from the cytoplasm to the extracellular environment. The deimination of arginine and agmatine leads to the generation of ammonia to counteract cytoplasmic acidification. The by-products of arginine and agmatine, putrescine and ornithine, respectively, are removed from the cell by an antiporter that also imports their cognate amino acid. In addition, enterococci decarboxylate tyrosine to its amine form, tyramine, consuming cytoplasmic protons to further protect against acidification. Carb-putresc, carbamoyl putrescine; Carb-PO_4, carbamoyl-phosphate.

In *Enterococcus hirae*, a common constituent of the mammalian and avian gut microbiota and an occasional pathogen of humans, a system comprised of an Na^+ pumping vacuolar (V_1V_0)-type ATPase and a K^+/H^+ antiporter is crucial for homeostasis under alkaline pH (126, 127). In fact, *E. hirae* has served as a model organism for studies of ion transport, bioenergetics, and copper homeostasis (128). Deletion of the cation/proton exchange system of *E. hirae* resulted in a near cessation of cell growth following the elevation of culture pH to 9.5 (129). The V_1V_0-ATPase couples the energy from ATP hydrolysis to pump Na^+ from the cell, generating a strong electrochemical potential ($\Delta\psi$) across the cell membrane (130). This large $\Delta\psi$ is likely needed to compensate for the unfavorable proton gradient (ΔpH) under alkaline conditions and drive the function of the K^+/H^+ antiporter to bring protons into the cell (126). Of note, the *ntp* operon coding for the V_1V_0-ATPase is conserved among enterococci. In addition, many *Enterococcus* spp. appear to encode a homolog of the extracellular sigma factor σ^w, which has been previously associated with the regulation of genes involved in adaptation to alkaline shock (131). However, it is also possible that alkaline shock causes an arrest in cell wall synthesis, thereby triggering σ^w activation (132). Identification of additional mechanisms of survival in an alkaline environment, as well as more detailed characterization of transcriptional regulation in response to alkaline pH, will surely reveal the mechanisms that allow the enterococci, among the neutralophiles, to survive and grow at unusually elevated alkaline pH.

Acid stress. The maintenance of ΔpH is also critical for acid tolerance. Unlike under alkaline conditions, the ΔpH is primarily maintained through proton extrusion. Proton extrusion in *E. hirae* is carried out by the F_1F_o-ATPase. Unlike the F-type ATPase of gammaproteobacteria such as *E. coli*, the *E. hirae* F-type ATPase operates in reverse to hydrolyze ATP and pump protons out of the intracellular medium (Fig. 4). The activity

of the F-ATPase increases greatly under low-pH conditions, indicating that it is a major contributor to the survival *E. hirae* at low pH (121).

Buffering the cytoplasmic environment is another mechanism of acid tolerance that has been characterized in *E. hirae*. Specifically, arginine (ADI) and agmatine (AgDI) deiminase pathways counter cytoplasmic acidification through the production of ammonia (133, 134). The routes for catabolism of these two amino acids are similar (Fig. 4). Arginine and its decarboxylated derivative agmatine are deiminated to L-citrulline and carbamoyl-putrescine, respectively, liberating free ammonia that can bind a proton yielding NH_4^+ and raising the cytoplasmic pH (135). Carbamoyl-putrescine and L-citrulline are then phosphorylated into to carbamoyl-phosphate. Carbamoyl-phosphate is metabolized by pathway-specific carbamate kinases, yielding ATP, CO_2, and another molecule of ammonia that can further buffer the cytoplasmic pH (133, 136). The by-products ornithine and putrescine, from the ADI and AgDI pathways, respectively, are removed from the cell by arginine-ornithine or putrescine-agmatine antiporters (137, 138). Notably, the ADI pathway can function to produce ammonia from arginine at constant pH values as low as pH 3.5, which is 1.5 pH units below the minimal pH permissible for growth of *E. hirae* in complex medium (133). Remarkably, ammonia production by the ADI system prevented loss of cell viability when cells were incubated in pH 2.5 buffered salt solution containing arginine, an otherwise lethal acid challenge (134). The biochemistry of the AgDI pathway has been studied in *E. faecalis* (135, 136, 139, 140), but its specific contribution to acid tolerance has not been established. However, the *E. faecalis* AgDI system is able to buffer the external pH and likely contributes to acid tolerance (140).

Amino acid decarboxylation is also thought to contribute to acid tolerance as decarboxylase pathways are induced at low pH (141). These systems involve decarboxylation of the amino acid to its cognate amine and then exchange of the amine for its amino acid precursor (Fig. 4). This process consumes a proton, lending to its cytoplasmic buffering capacity. Similar to the ADI pathway above, the tyrosine decarboxylase (TDC) pathway of *E. faecium* E17 was shown to exert a protective effect against acute acid challenge through neutralization of the external pH and maintenance of the intracellular ΔpH (142).

Regulation of the ADI and AgDI systems is complex. The AgDI operon in *E. faecalis* is subject to carbon catabolite repression (CCR) by the nutrient-sensing transcriptional regulator CcpA as well as by a CcpA-independent pathway involving the mannose phosphotransferase system (PTS) (140). The *E. faecalis* AgDI operon is also activated by the agmatine-sensing transcriptional regulator AguR (140). In the oral commensal *Streptococcus gordonii*, regulation of the ADI system is also subject to CCR by CcpA as well as by the arginine-sensing Fnr/Crp family transcriptional activator ArcR (143). Although not directly assessed, a similar regulatory mechanism seems to exist in enterococci. More specifically, arginine deimination was shown to be under CCR control in *E. faecalis* (144), and a Fnr/Crp family transcriptional regulator homolog appears to be present in the enterococcal ADI operon.

Summary and perspectives. A strong foundation has been established to understand the molecular mechanisms contributing to acid and alkaline stress tolerance pathways in the enterococci (Fig. 4). Studies of F-ATPase and deaminases in members of the enterococci have provided paradigms for understanding the function of these enzymes in other organisms. In addition, the regulation of the acid-adaptive response is relatively well defined, particularly the complex transcriptional regulation of AgDI deaminase by the CcpA catabolite repressor and the agmatine sensing regulator AguR (140). It is likely that further studies of the transcriptional regulation of acid and alkaline stress response stimulons will help uncover additional levels of transcriptional regulation that contribute to the robustness of the enterococci. In addition, the contribution of these pH-adaptive pathways to host colonization and pathogenesis has not been examined. Such studies may prove particularly informative in environments such as the intestine, where a broad pH range characterizes distinct anatomical regions.

Osmotic Stress

Osmoregulation allows cells to survive sustained or transient fluctuations in environmental osmotic pressure. Drastic changes in osmotic pressure are commonly encountered by enterococci as they move from the intestines to oligotrophic water sources. In addition, uropathogenic enterococci encounter osmotic stress in the bladder during cycles of urine concentration and dilution. If not properly managed, exposure to high external solute concentration or hyperosmotic stress can cause a detrimental concentration of cytoplasmic contents and a reduction in turgor pressure. Since bacteria generally lack active water transport systems, cells accumulate compatible solutes through transport or synthesis to counter the imposed water efflux during periods of hyperosmotic stress (145). These so-called compatible solutes accumulate to high intracellular concentrations without apparent disruption of essential cellular processes (146). Osmoprotective solutes are typically organic compounds, such as the disaccharides (e.g., trehalose), amino acids (e.g., glutamine, glutamate, and proline), or quaternary amines (e.g., glycine betaine), but may also be inorganic ions such as potassium (146). In addition, bacteria possess mechanosensitive aquaporins to control the efflux of water from the cell (147). Aquaporins are critical for adaptation during hypoosmotic stress (i.e., low external solute concentration) as they mitigate the effects of water influx that would otherwise result in considerable pressure and damage to the cell membrane and cell wall (148). Given the potential importance of osmoadaptation in the ecology of the enterococci, the detailed study of this process is an important endeavor.

Transcriptional profiling of *E. faecalis* V583 upon exposure to 6.5% NaCl has shed some light on osmoregulation in the enterococci. A number of active transport systems for compatible solutes are induced, including the *kdp* operon encoding a K^+-importing ATPase and two amino acid transporters for glycine-betaine, carnitine, choline, and proline (149). Glycine-betaine uptake is osmoprotective in *E. faecalis* ATCC 19433 and enhances growth in the presence of high salt concentrations (150). Several more osmotic pressure-specific adaptive responses are also transcriptionally activated, including the *ntp* operon encoding the V-type, Na^+-exporting ATPase, the fatty acid biosynthesis pathway, and the cell wall polysaccharide biosynthesis operon *epa* (149). Mutational analysis confirmed that cell envelope modification by Epa provides osmoprotection from high levels of NaCl (149). Of the major known osmoregulators, *E. faecalis* has homologs to the highly conserved *kdpED* two-component system (TCS) whose activity is also thought to be regulated by physical parameters associated with osmotic stress, such as turgor pressure and ionic strength (146). The KdpED TCS might also be interwoven into larger stress networks in *E. faecalis* through interaction with sugar PTS components, universal stress proteins, or membrane proteins acting as ligand binding adaptors, as seen in other pathogens (151). The hyperosmotic transcriptome also included genes encoding the protein chaperones DnaK and GroEL/GroES, which contribute to diverse stress tolerance processes (149). Thus, the transcriptional response to hyperosmotic stress induces both specific and general tolerance mechanisms.

Summary and perspectives. The mechanisms by which *E. faecalis* and the enterococci in general respond to osmotic stress are still poorly defined. Transcriptional analysis has identified candidate pathways that include active ion transport systems, molecular chaperones, and factors affecting the physiochemical properties of the cell envelope (149). With the exception of the *epa* operon (149), direct experimental evidence to confirm the contributions of each pathway to osmoadaptation is still needed. In addition, osmotic adaptation is known to contribute strongly to the ability of uropathogenic *E. coli* to stably colonize the murine urinary track (152). However, nothing is known in regard to regulation of these systems or how they might impact pathogenicity of enterococci during urinary tract infections.

Metal Stress

Transition metals, particularly Fe, Mn, Zn, and Cu, are critical cofactors for the

function of numerous bacterial enzymes involved in redox balance, proteolysis, and central metabolism. Animal hosts, from insects to vertebrates, have evolved a branch of the innate immune response called nutritional immunity, which comprises a class of immune proteins that sequester these essential metals, to limit microbial growth. For example, in mammals, transferrin and hemoglobin keep Fe nearly inaccessible to bacterial pathogens in the bloodstream (153). In addition, the immune system also exploits the toxicity of metal accumulation in the bacterial cytosol to regulate bacterial growth (154). For example, macrophages take advantage of this metal toxicity by pumping copper into the phagocytic compartment to combat bacterial pathogens (155). In order to maintain intracellular metal levels sufficient for balanced growth while avoiding toxic buildup, i.e., metal homeostasis, bacteria have evolved sophisticated systems to regulate the uptake and efflux of metals. This section focuses on Cu, Mn, and Fe homeostasis in the enterococci.

Copper stress. Compared to iron and manganese, copper (Cu) serves as a cofactor for a limited range of bacterial enzymes, such as cytochrome *c* oxidase (156), certain classes of superoxide dismutase (40), and nitrite and nitrous oxide reductases (157, 158). In fact, members of the *Firmicutes*, including *Enterococcus,* do not encode any of these Cu^{2+}-containing enzymes. Thus, copper homeostatic mechanisms in enterococci seem to exist to prevent copper intoxication. Copper toxicity results from the ability of copper to participate in redox chemistry, leading to the generation of ROS, or to compete with other metal cofactors causing enzyme mismetallation (159, 160).

Copper homeostasis has been most extensively researched in *E. hirae* and has served as a model for this process among bacteria. Of note, the *E. hirae* genes and regulators discussed below are highly conserved across the enterococcal genus (Fig. 2; see also Table S1 in the supplemental material). In *E. hirae*, intracellular copper pools are controlled by a four-gene cluster called the *cop* operon, consisting of the copper transporting ATPases *copA* and *copB*, the chaperone *copZ*, and the transcriptional regulator *copY* (Fig. 5). Notably, environmental copper primarily exists in the soluble, oxidized Cu^{2+} form, but insoluble Cu^{+} appears to be the preferred substrate of copper ATPases such as CopA and CopB. This fact argues for the presence of an extracellular copper reductase. In support of this, cultures of *E. hirae* grown in the presence of exogenous copper lost the characteristic blue appearance associated with oxidized Cu^{2+} within several hours. This activity is likely due to an extracellular or membrane-bound copper reductase that has yet to be identified (156).

The copper ATPases are responsible for the flux of copper in and out of the cell. According to the current model, CopB is a copper-extruding transporter needed to keep copper from accumulating to toxic levels in the cytoplasm. The metal-extruding capacity of CopB has been experimentally validated through *in vitro* enzymatic assays and cell labeling assays (161, 162). CopA is thought to function in copper uptake during periods of copper limitation. The evidence for this is indirect and is based on the observation that deletion of *copA* resulted in poor growth when copper was limited in the culture medium by the addition of copper chelators. In addition, the Δ*copA* strain was also more resistant to the antibacterial effects of exogenous silver, which is also transported by the copper ATPase family, suggesting that *copA* may also transport silver (161). It is also possible that copper import by CopA may support molybdenum cofactor biosynthesis, a process that requires copper in plants (163). The molybdenum cofactor is essential for *de novo* purine synthesis, a pathway present in the enterococci. Future work will be required to determine the role of the *copABZY* operon in copper homeostasis and, perhaps, in the support of purine biosynthesis.

CopY is a copper-sensitive transcriptional regulator modulating transcription of the *cop* operon to ensure copper balance. Under conditions of copper excess, cytoplasmic Cu^{+} binds to the CopZ chaperone. The copper-bound CopZ routes copper to CopY, inducing expression of *copA* and *copB*. The enhanced expression of the CopB ATPase and the CopZ chaperone works to extrude and sequester the excess copper until the intracellular levels drop sufficiently to return the system to its original suppressed state. In addition, Cu^{+} competes with Zn^{2+} to bind CopY (Fig. 5). The mismetallation of CopY

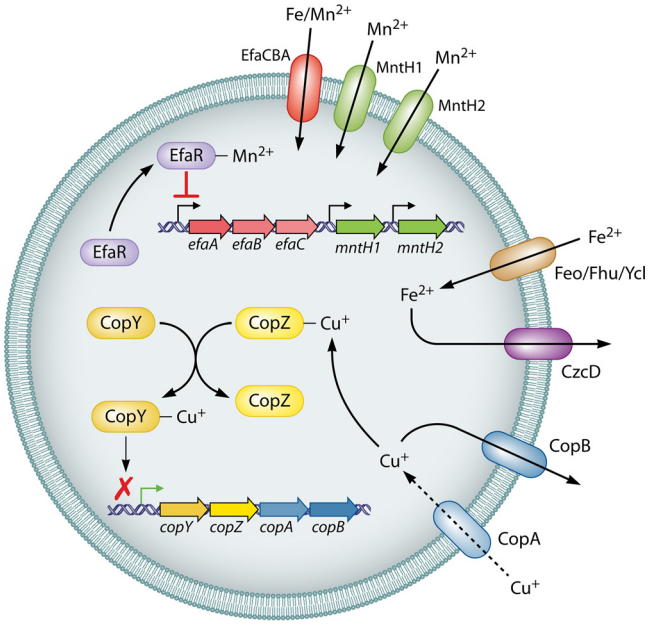

FIG 5 Metal transport and homeostatic regulation. In *E. hirae*, import and export of Cu^{+} are controlled by the *cop* operon. The ATPases CopB and CopA facilitate the export and uptake of Cu^{+}, respectively. Under conditions of copper excess, the metal binding chaperone CopZ transfers its bound Cu^{+} to the CopY transcriptional regulator, blocking its DNA binding activity and inducing the *cop* operon to prevent toxic accumulation of Cu^{+}. CopA is believed to be responsible for Cu^{+} import under Cu^{+} limitation. In the current model, Mn^{2+} is brought into the cell by several cation transporters, including EfaCBA, MntH1, and MntH2. Under conditions of Mn^{2+} limitation, these transporters are actively transcribed. Once Mn^{2+} accumulates to sufficient levels, it binds to the EfaR transcriptional regulator, leading to reduced Mn^{2+} transporter expression and Mn^{2+} uptake. Under conditions of low external Fe^{+}, intracellular Fe^{2+} levels appear to be maintained by the Feo, Fhu, and Ycl multisubunit active uptake systems. The Nramp/MntH family of cation transporters may also have multication specificity and could also participate in Fe^{2+} uptake. When Fe^{2+} reaches toxic levels, *E. faecalis* activates expression of the CzcD cation efflux pump to remove excess Fe^{2+}.

by Zn^{2+} instead of Cu^{+} hinders DNA binding and repression, leading to the activation of the *cop* operon when copper is limiting (164). CopZ levels are also controlled by proteolysis although the identity of the corresponding protease of CopZ is not known. However, an enzyme responsible for CopZ degradation was blocked by serine protease inhibitors and therefore appears to belong to the serine protease family (165). The CopZ chaperone was also shown to interact with CopA *in vitro*, suggesting that CopZ also functions to accept and sequester incoming Cu^{+} ions (166). In addition, CopZ is more labile in its Cu-bound form (165), and the solvent-exposed Cu cofactor of CopZ presents a redox-active catalyst for ROS-generating Fenton-like chemistry (164, 167). Thus, regulation of copper homeostasis by the Cop proteins is calibrated to minimize intracellular buildup of copper to prevent generation of cytoplasmic ROS.

Manganese stress. Unlike copper, manganese (Mn) is an essential metal cofactor in bacteria. Enzymes requiring manganese for catalysis are involved in key steps of central carbon metabolism, namely glycolysis and synthesis of the nucleotide second messengers cyclic-di-GMP and (p)ppGpp (168). The availability of free Mn is especially relevant to lactic acid bacteria such as the enterococci as Mn mitigates ROS stress, which is an intrinsic product of fermentative metabolism of lactic acid bacteria (169, 170). This is accomplished through the Mn-dependent superoxide dismutase (SOD) enzyme, as discussed earlier, and through the substitution of Mn for Fe as a cofactor in metalloenzymes, which limits the deleterious effects of Fe-mediated Fenton chemistry (171, 172). In *E. coli*, the mismetallation of iron-containing enzymes with manganese poisons *de novo* heme biosynthesis and respiration. However, the lactic acid bacteria lack both cellular processes and, thus, can maintain an exceptionally high intracellular Mn/Fe ratio (170, 173, 174). Thus, there is an apparent trade-off between metabolic versatility and oxidative stress tolerance in lactic acid bacteria.

Manganese uptake is a key point at which cellular Mn levels are regulated. Bacterial high-affinity Mn transporters come in three flavors: ATP-binding cassette (ABC) permeases, Nramp (natural resistance-associated macrophage protein) H^+/Mn transporters, a class of uptake systems also found in eukaryotes, and less common P-type transporters (175, 176). The link between Mn acquisition and virulence was first established with the realization that virtually all bacterial pathogens possess at least one Nramp-homologous transporter: a macrophage-associated Mn transporter that confers natural resistance to infection by promoting proinflammatory responses and oxidative burst (177). Mn transporters can impact bacterial virulence in two ways: (i) by promoting bacterial growth in metal-depleted tissues and (ii) by interfering with macrophages and the innate immune system. In *Firmicutes*, the role of Mn transporters has focused almost exclusively on ABC-type Mn permeases. In enterococci and streptococci, these permeases have been linked to infective endocarditis (IE) (178–181).

The core genome of *E. faecalis* encodes two Nramp-type transporters, MntH1 and MntH2, and one ABC transporter, EfaCBA (182). Earlier studies revealed that the *efaCBA* operon is expressed as a single polycistronic transcript and is strongly induced during Mn^{2+} limitation; this induction is reversed only by the addition of Mn^{2+} but not by other metal cofactors, initially suggesting that EfaCBA functions specifically in Mn^{2+} uptake (183). In *E. faecalis*, EfaA was first discovered as a major antigen recognized by the sera of patients with enterococcal IE (183, 184) and has been used as an immunodiagnostic tool to discriminate *E. faecalis* from other IE-causing pathogens (185). Virulence of the Δ*efaA* mutant in the OG1RF background strain was attenuated in a mouse peritonitis model, underscoring its relevance during systemic infection (185). The *efaCBA* operon is regulated by EfaR, a homolog of the DtxR metalloregulatory protein from *Corynebacterium diphtheria*. EfaR showed enhanced binding to the *efaCBA* operon promoter region in the presence of Mn^{2+} but also of several other divalent cations (183). A more detailed examination of EfaR regulon by microarrays showed that EfaR also controls the expression of the two Nramp-type transporters, MntH1 and MntH2, and three adjacent genes forming a cationic ABC transporter (Fig. 5). In a recent study, the *efaCBA*, *mntH1*, and *mntH2* genes were systematically inactivated in the OG1RF strain, generating strains lacking one (Δ*efa*, Δ*mntH1*, and Δ*mntH2* strains), two (Δ*efa* Δ*mntH1*, Δ*efa* Δ*mntH2*, and Δ*mntH1* Δ*mntH2* strains), or all three (Δ*efa* Δ*mntH1* Δ*mntH2* strain) transporters (186). Loss of *efaCBA* alone impaired growth in serum or in the presence of calprotectin, a Mn/Zn-sequestering protein of the S100 family that accounts for more than 40% of the total protein content of neutrophils. The inactivation of *mntH2* exacerbated the phenotypes of the Δ*efa* mutant and drastically reduced the intracellular Mn content, suggesting that EfaCBA and MntH2 are the primary Mn transporters of *E. faecalis*. While only the Δ*efa* Δ*mntH1* Δ*mntH2* triple mutant showed virulence attenuation in the *G. mellonella* invertebrate model, both the Δ*efa* Δ*mntH2* double and Δ*efa* Δ*mntH1* Δ*mntH2* triple mutant strains were virtually avirulent in a rabbit IE and in a murine catheter-associated urinary tract infection (CAUTI) model (186).

Iron stress. Like Mn, iron (Fe) is an essential micronutrient required for the growth of nearly all bacteria (187). Iron primarily exists in the two redox states, the ferrous (Fe^{2+}) form or the ferric (Fe^{3+}) form. The ability of Fe ions to readily interconvert between Fe^{2+} and Fe^{3+} forms makes iron an ideal biocatalyst for redox enzymes or as an electron carrier involved in respiration, the tricarboxylic acid (TCA) cycle, and DNA replication (187). Although Fe is one of the most abundant surface metals on earth, its biological availability is limited because it exists in the highly insoluble Fe^{+3} form under aerobic conditions and at physiological pH. Similarly, in animal hosts free Fe ions are tightly complexed to proteins, most notably heme-containing proteins in vertebrates. High-affinity transporters and small molecules known as siderophores, with some of the highest binding affinities for Fe observed in nature (188), are used to counteract Fe limitation in numerous environments. Although almost universally essential in bacteria, free Fe ions pose a significant threat to bacterial cells if allowed to interact with H_2O_2, leading to the generation of more reactive and lethal hydroxyl radicals. Because of the

essential but potentially harmful nature of Fe, bacteria have evolved complex homeostatic mechanisms to balance their need to efficiently scavenge but also prevent the toxic accumulation of cellular Fe.

The transcriptional responses of *E. faecalis* OG1RF to both Fe excess and limitation have been determined. When compared, these often-divergent transcriptional profiles outline the molecular and regulatory machinery that appears to be critical for maintaining Fe levels compatible with balanced growth and survival. Growth of *E. faecalis* in a semidefined medium containing 0.5 mM $FeCl_3$, in a 1:1 complex with the chelator nitrilotriacetic acid to enhance stability and solubility, results in a 6-fold increase in intracellular Fe levels (189). Under these conditions, three functional classes of genes involved in iron stress were differentially expressed. The first functional class is composed of genes related to Fe uptake and efflux. As expected under Fe excess, several uptake systems were transcriptionally repressed. These Fe transport systems include the *fhu* ferrichrome ABC transporter, *feo* family ferrous iron transporters, the Nramp Mn^+/Fe^+ transporter *mntH*, and the *ycl* iron compound transporter. In addition, the cation efflux family protein *czcD* was induced (189). A second group of genes corresponding to the management of redox stress that includes *sodA*, *katA*, *trx*, *ohr*, and *msrA* was activated 2-fold or greater under Fe excess (189). The third functional group was comprised of the Fur family transcriptional regulators *perR* and *zurR*, both of which are induced under Fe excess conditions (189). As discussed above, PerR becomes highly sensitive to redox stress when complexed with Fe^{2+}, resulting in activation of oxidative stress genes like *katA* and *ohr* in *E. faecalis* (88, 93). Together, these expression changes presumably work to protect cells by blocking Fe uptake, excreting excess free Fe ions, scavenging ROS, and repairing any resulting oxidative damage.

The blood of vertebrate animals can be severely limiting for bacterial growth because Fe ions are tightly complexed with the iron binding proteins hemoglobin, myoglobin, or transferrin (190). Growth in human blood, therefore, represents a logical comparison to confirm that transcriptional alterations to the functional gene classes described above are biologically meaningful. In other words, genes functioning to maintain Fe homoeostasis should show opposing transcriptional trends in response to Fe excess and limitation, respectively. This was generally true for genes with functional annotations for Fe ion uptake and efflux. The previously mentioned *feo*, *ycl*, and *mntH* transporters were transcriptionally activated in blood, while the *czcD* efflux protein gene was repressed (169, 189). However, in a comparison of the groups of genes involved in redox stress management and transcriptional regulation, *perR* and *zurR*, similar expression trends were seen between growth in Fe excess and that in blood (169, 189). The latter two functional groups could, therefore, represent transcriptional responses that are important but not specific for Fe stress tolerance. Moreover, blood is a complex biological fluid in which other factors like host immune cells may activate redox stress responses despite Fe limitation.

Summary and perspectives. The *cop* operon of *E. hirae* remains one of the most well understood systems for Cu homeostasis in bacteria, both in terms of mechanism and regulation. In future investigations, it will be interesting to evaluate if Cu proves an essential component in molybdenum biosynthesis in enterococci and other prokaryotes, thereby providing an explanation for the biological significance of CopA. Additionally, little is known about the potential virulence contribution of the *cop* genes. Copper-homeostatic mechanisms are highly conserved across bacterial genera, suggesting that this machinery predates the emergence of enterococci and is therefore too ancient to have evolved to counter host innate immune defenses (4, 159). In spite of this, it would be interesting to examine the contribution of this system to the infectious process for several reasons. Copper intoxication is critical for host phagocytes to eradicate bacterial pathogens, and Cu resistance was shown to be a major virulence determinant in *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, and *Streptococcus pneumoniae*, all of which rely on the ability to survive or replicate inside macrophages for their pathogenic potential (191–193). *E. faecalis* also exhibits

the ability to survive for prolonged periods in macrophages, which may be used as a mechanism for systemic spread (56).

Global transcriptional profiling experiments have laid the foundation for understanding the genetic pathways and regulation of Fe homeostasis (169, 189). The functional role of these genes in maintaining Fe homeostasis has not been confirmed experimentally. However, given the high level of functional conservation of many Fe homeostatic mechanisms across bacteria, there is some confidence that these genes and regulators are important (187). Regulation of these responses may be based, at least in part, on the activity of PerR and ZurR (189). In *B. subtilis*, control of Fe homeostasis is governed by the Fe-sensing transcriptional regulator Fur (194). The gene *EF1525* represents a strong amino acid sequence homolog with 71% identity over the full-length *B. subtilis* Fur protein (BSU23520). This Fur homolog could coordinate similar transcriptional responses to extremes in Fe availability. Posttranslational control of Fur activity by Fe metalation level, rather than transcriptional control, could explain the absence of Fur from transcriptional profiling experiments.

The documented importance of Cu^{+}, Fe^{+}, and Mn^{2+} stress and homeostatic mechanisms for virulence in other bacterial pathogens suggests that an in-depth understanding of the structure and regulation of these pathways may prove valuable for drug design. In fact, a recent study examining these types of structure-function relationships has provided detailed mechanistic insight into ability of zinc ions to block the activity of a manganese uptake system critical for nasopharyngeal colonization and virulence in *S. pneumoniae* (195, 196). The finding that a triple mutant *E. faecalis* strain lacking all three Mn transporters was virtually avirulent in two mammalian models (197, 198) serves as a strong indication that pathways associated with Mn homeostasis are promising targets for the development of new antimicrobials to combat *E. faecalis* infections. However, the importance of Mn transport systems to other pathogenic enterococci, in particular *E. faecium*, remains to be explored.

Nutritional Stress

In their natural environment, bacteria are subjected to sudden and drastic fluctuations in nutrient source and availability. As a consequence, bacteria have evolved complex and interconnected regulatory networks to sense and integrate both internal and external metabolic cues. These regulatory networks control large gene subsets needed to utilize diverse sources of carbon, nitrogen, sulfur, and phosphorus. When it comes to nutrient availability and source, enterococci are extremely versatile organisms. For example, *E. faecalis* is able to survive prolonged periods under oligotrophic conditions and to obtain energy from a variety of carbon sources (37, 199). This versatility helps to explain, at least in part, the ability of enterococci to colonize a wide variety of hosts as well as oligotrophic environments, such as the majority of aquatic environments. Members from this genus are able to transport and ferment a broad range of carbohydrates. A core set of 13 sugars are metabolized by all studied species of *Enterococcus*, with 31 additional sugars metabolized by one or more species. A more comprehensive review of the topic can be found elsewhere (199).

In *Firmicutes*, the control of key metabolic intersections is governed by several nutrient-sensing global regulators, including CcpA and CodY and the nucleotide second messenger (p)ppGpp, the effector molecule of the stringent response (SR) (200, 201). Importantly, these three regulatory pathways are known to control the expression of genes critical for the pathogenic and virulence potentials of numerous Gram-positive pathogens (202–204). The next section will focus on (p)ppGpp as its metabolism and downstream effects have been recently characterized in *E. faecalis* and will also relay pertinent features associated with CodY and CcpA, which are likely to contribute to nutrient stress tolerance and virulence in enterococci.

(p)ppGpp and the stringent response. The stringent response is a conserved stress response mechanism in most eubacteria triggered by rapid and robust accumulation of two nucleotide second messengers, guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp). Together, these two nucleotides are referred to as

(p)ppGpp. The accumulation of (p)ppGpp broadly remodels cellular physiology to transition rapidly growing bacterial cells to a state of slow growth or stasis. This occurs through a general repression of macromolecular biosynthesis, activation of nutrient biosynthesis and uptake, and activation of various stress responses (205). These broad physiological alterations occur both at the transcriptional level and through direct inhibition of enzymes involved in DNA replication, GTP biosynthesis, and translational initiation and elongation (201, 206). Although initially characterized as a nutritional stress response, (p)ppGpp and the stringent response enhance tolerance to an extensive array of stresses and control the expression of virulence traits (203, 206).

Metabolism of (p)ppGpp in bacteria is carried out by a functionally diverse and widely distributed family of RelA/SpoT homolog (RSH) enzymes, named after the two enzymes mediating (p)ppGpp metabolism in *E. coli*. In *Firmicutes*, the bifunctional (p)ppGpp synthase/hydrolase Rel, also known as Rsh or RelA, is the primary enzyme responsible for the metabolism of (p)ppGpp. In addition to Rel, there are up to two small monofunctional synthases called RelQ or RelP that participate in (p)ppGpp metabolism in *Firmicutes* (207, 208). In *E. faecalis*, the metabolism of (p)ppGpp is carried out by the bifunctional Rel (Rel_{Ef}) and weak (p)ppGpp synthase $RelQ_{Ef}$. Rel_{Ef} is the enzyme necessary for induction of the stringent response following amino acid starvation (209, 210). $RelQ_{Ef}$ did not contribute significantly to (p)ppGpp accumulation under amino acid starvation conditions but, rather, contributed to the maintenance of basal (p)ppGpp pools under nonstressed conditions (209) and to a timely activation of the stringent response (211).

Two hallmarks of the stringent response are the strong repression of macromolecular biosynthesis genes and the simultaneous activation of nutrient biosynthesis and transport genes to maintain continued growth under nutritional limitation. Microarray analysis of *E. faecalis* cells treated with mupirocin, an isoleucine tRNA synthase inhibitor that strongly induces (p)ppGpp accumulation, results in the repression of a large percentage of genes involved in ribosome assembly and biogenesis, chromosomal DNA replication, and RNA modification and maturation (211). In addition, there is increased expression of numerous nutrient transporters, most of which are predicted to function in amino acid and oligopeptide uptake (211). However, very few genes involved in the *de novo* synthesis of amino acids are activated in *E. faecalis*, a prominent transcriptional signature of the stringent response in other bacterial species (203, 211). This seems to reflect the lack of several amino acid biosynthesis pathways and subsequent auxotrophies, such as branched-chain amino acid (BCAA) biosynthesis, in *E. faecalis* (212) that are likely satisfied by the host diet or microbial cross-feeding interactions.

Phenotypic characterization of *E. faecalis* OG1RF derivatives with deletions of rel_{Ef}, $relQ_{Ef}$, or both reveals a complex and nuanced relationship between (p)ppGpp levels, starvation survival, and metabolic homeostasis. The absence of rel_{Ef}, alone or in combination with $relQ_{Ef}$ has a detrimental impact on the volume, architecture, and long-term viability of nutrient-limited biofilms (213). An inability to maintain proper metabolic homeostasis is observed only in the complete absence of (p)ppGpp (Δrel_{Ef} $\Delta relQ_{Ef}$ mutant, or (p)ppGpp^0 strain) (214). This was exemplified by an uncontrolled accumulation of intracellular GTP to inhibitory levels in the presence of exogenous guanosine and a shift from homolactic to heterolactic fermentation and the overproduction of H_2O_2 as a metabolic by-product (214). These results indicate that it is just not the stringent response but more subtle changes in (p)ppGpp pools, well below the levels observed during the stringent response, that are needed for metabolic homeostasis in *E. faecalis* (209, 214). Recent enzymatic characterization of RelQ from *E. faecalis* revealed several enzymatic features new to RSHs, including the utilization of GMP for synthesis of pGpp. *In vitro* analysis revealed that pGpp strongly inhibits the activity of *E. faecalis* enzymes involved in GTP biosynthesis and, to a lesser extent, transcription of *rrnB* by *E. coli* RNAP (215). The ability of pGpp to exert complex, target-specific effects on processes known to be modulated by (p)ppGpp suggests the existence of pGpp-mediated signaling in enterococci and likely other bacteria, such as streptococci, whose small synthetases also exhibited pGpp synthetic capacity *in vitro* (215).

The virulence of *E. faecalis* strains with defects in (p)ppGpp metabolism has been explored in some detail (198, 209, 211, 216). The (p)ppGpp^0 double mutant strain showed attenuated virulence in two invertebrate models (*Caenorhabditis elegans* and *G. mellonella*) and in a rabbit abscess model (209, 211, 216). However, virulence of the Δrel_{Ef} strain, which like the (p)ppGpp^0 strain is unable to mount the SR, in these *in vivo* models was not affected. Even more surprising, the Δrel_{Ef} single mutant but not the (p)ppGpp^0 strain showed attenuated virulence in a rabbit endocarditis model (198). It follows that while the double mutant, the (p)ppGpp^0 strain, is completely unable to synthesize (p)ppGpp, basal (p)ppGpp pools are about 4-fold higher in the Δrel_{Ef} strain due to the activity of RelQ (214). Thus, the picture that emerged from these studies is that the metabolic control exerted by basal (p)ppGpp pools is more important during infections than the semidormancy state characteristic of the SR.

While the stringent response and the enzymes involved in (p)ppGpp metabolism in *E. faecium* have not been characterized, a recent study identified the emergence of a single missense mutation in the Rel enzyme (termed RelA in the original publication) of a case of *E. faecium* bacteremia that persisted for 26 days despite appropriate antibiotic therapy (217). The missense mutation (L152F) in the *rel* gene was first detected 3 days after the start of antibiotic therapy and resulted in elevated baseline levels of ppGpp without affecting total (p)ppGpp pools upon activation of the stringent response with mupirocin. Although the *rel* mutant remained susceptible to linezolid and daptomycin during planktonic growth, it demonstrated tolerance to high doses of both antibiotics when grown in biofilms. These observations suggest that increased baseline production of the ppGpp alarmone promote *E. faecium* antibiotic tolerance (persistence) *in vivo*.

CodY and CcpA. CodY is a nutrient-sensing metabolic regulator in *Firmicutes*. Initially identified in *B. subtilis*, CodY represses the transcription of genes needed for amino acid anabolism and degradation of extracellular macromolecules but also activates carbon overflow pathways for acetate and acetoin synthesis under carbon- and nitrogen-rich conditions (200). CodY is a dimeric DNA binding protein whose transcriptional regulatory activity is enhanced by BCAA (200) and, with the apparent exception of streptococci (218), GTP (Fig. 6). In doing so, CodY couples carbon and nitrogen availability to the flow of carbon and nitrogen through the cell (200).

The capacity of CodY to sense intracellular GTP levels creates a direct link to the stringent response (Fig. 6). In *B. subtilis*, induction of the stringent response depletes GTP pools in both a direct and indirect manner. First, the rapid production of (p)ppGpp occurs at the expense of GTP. Second, the accumulation of (p)ppGpp during the stringent response further lowers GTP pools through repression of GMP kinase (Gmk) and hypoxanthine-guanine phosphoribosyltransferase (HprT) activity. Inhibition of Gmk and HprT blocks both the *de novo* and salvage pathways of GTP biosynthesis (219). In *E. faecalis*, (p)ppGpp specifically and strongly inhibited the activity of HprT (214) and one of the two Gmk enzymes (215) supporting a homologous mechanism for the repression of GTP biosynthesis (Fig. 6).

The relationship between (p)ppGpp and CodY has been shown to control virulence in several Gram-positive bacteria. For example, in *S. aureus*, the infectivity of a *rel* (*rel*$_{Sa}$) mutant was significantly attenuated in a murine model, a phenotype that was restored by inactivation of *codY* in the *rel*$_{Sa}$ mutant background (220). Similarly, inactivation of *rel* in *Listeria monocytogenes* (*rel*$_{Lm}$) leads to an avirulent phenotype in a murine listeriosis model, which could be restored by inactivation of *codY* in the *rel*$_{Lm}$ background (221). In *E. faecalis*, *in silico* analysis suggests that numerous genes activated during the stringent response contain CodY binding motifs, including amino acid biosynthesis, amino acid/oligopeptide transport, and stress tolerance genes. Inactivation of *codY* in the (p)ppGpp^0 background restored several phenotypes associated with the lack of (p)ppGpp in *E. faecalis* OG1RF, including the inability to grow in whole blood and virulence in *G. mellonella* (197). Therefore, the canonical association between (p)ppGpp and CodY shown in other Gram-positive species bacteria appears to be conserved in *E. faecalis*.

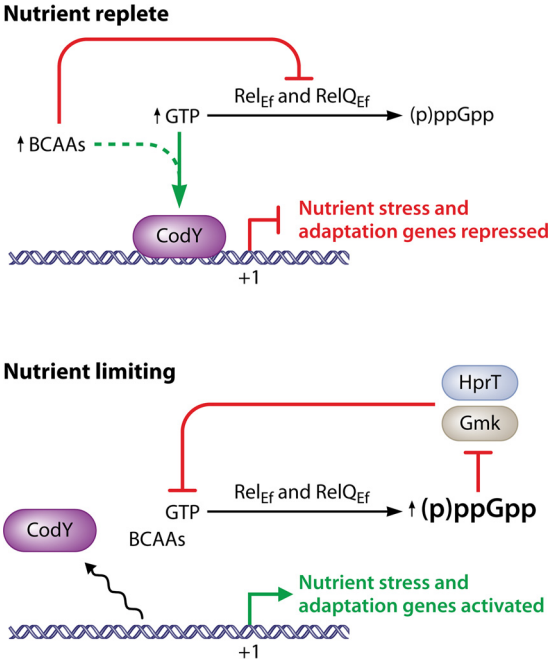

FIG 6 Regulation of nutrient stress tolerance. CodY and (p)ppGpp are key global metabolic regulators in *Firmicutes* that mediate broad transcriptional shifts and metabolic rearrangements in response to changing nutrient availability. During periods of sufficient nutrient availability, branched-chain amino acid (BCAA) and GTP levels remain high in the cell. Because BCAAs and GTP are corepressors of CodY, high levels of BCAAs and GTP keep nutrient stress adaptation genes under CodY repression when nutrients are abundant. High BCAA levels also maintain the bifunctional (p)ppGpp synthetase/hydrolase Rel_{Ef} in a hydrolytically active state, and, as a result, the cells display low intracellular pools of (p)ppGpp. When cells become nutrient limited, Rel_{Ef} shifts toward a synthetase-active state leading to (p)ppGpp accumulation and a precipitous drop in GTP levels through its direct consumption and inhibition of enzymes (HprT and Gmk) involved in the beginning steps of *de novo* GTP biosynthesis by (p)ppGpp. BCAA and GTP depletion also alleviates CodY regulation activity such that transcription of nutrient adaptation genes is enhanced.

CcpA is known to repress genes involved in the uptake and utilization of alternate carbon sources and, like CodY, activates genes for carbon overflow in the presence of the preferred carbon substrates by binding to catabolite-responsive element (CRE) sites in gene promoter regions (222). Regulation of CcpA activity is multifactorial. DNA binding by CcpA is enhanced through direct interaction with the glycolytic intermediates fructose-1,6,-bisphosphate (FBP) and glucose-6-phosphate and the protein HPr, a component of the sugar phosphotransferase system (PTS) (223, 224). However, in *B. subtilis* and *S. mutans*, CcpA is also an activator of stress responses and BCAA biosynthetic genes (225, 226). Currently, genes known to be under CcpA control in enterococci are limited to the divergent citrate metabolism operons, L-serine dehydratase, and two genes composing dihydroxyacetone kinase (227, 228). These operons collectively catabolize citrate, serine, and glycerol to pyruvate.

Summary and perspectives. A combination of transcriptional, proteomic, and biochemical studies revealed that enterococci, when faced with severe nutrient limitation, mount a classic stringent response characterized by repression of macromolecular biosynthesis and activation of stress survival pathways (209–211). The bifunctional (p)ppGpp synthetase/hydrolase Rel_{Ef} is the primary enzyme responsible for stringent response induction and is critical for long-term cell viability in starved biofilms (209–211). However, phenotypic characterization has proven that the presence of (p)ppGpp, even at low levels, is key to controlling metabolic homeostasis in *E. faecalis* (214). As result, a critical area for future study is to define the downstream regulatory cascade that lies between (p)ppGpp and its pleiotropic effects on growth and survival in *E. faecalis*, which includes the nutrient-sensing regulator CodY. In addition, the metabolic dysregulation observed in the (p)ppGpp null strain of *E. faecalis* suggests that (p)ppGpp

impacts the levels of central metabolic cues outside guanine nucleotide levels. However, besides GTP, little is known regarding the broader metabolic alterations that occur over the large dynamic range of intracellular (p)ppGpp as cells transition between states of rapid growth and dormancy.

Antibiotic Stress

With the advent of the antibiotic era beginning with the large-scale use of chemotherapeutics like salvarsan, sulfonamides, and penicillin, successful bacterial pathogens were either inherently resistant or rapidly acquired resistance against these compounds (229). Enterococci are notorious for their combination of intrinsic and acquired resistance to clinically relevant antibiotics, most notably β-lactams, aminoglycosides, and glycopeptides. It should be noted that intrinsic and acquired antibiotic resistances have distinct origins. Intrinsic resistance is mediated by genetic determinants encoded within the core genome of enterococci, while acquired resistance is mediated by genetic determinants obtained through horizontal gene transfer or by mutations selected for in the presence of antibiotic pressure. Intrinsic resistance also includes a phenomenon known as antibiotic tolerance, defined as tolerance to killing by concentrations of antibiotics near their MICs. Although antibiotic resistance does not directly contribute to virulence in enterococci, it does greatly enhance risk for colonization and subsequent infections in patients undergoing an antibiotic treatment regimen (230). The topic of acquired antibiotic resistance in enterococci has been covered in depth previously (5, 231–233); thus, this section will instead focus on conserved mechanisms for intrinsic antibiotic resistance and antibiotic tolerance in enterococci.

***β*-Lactams.** β-Lactam antibiotics bind and irreversibly inhibit the action of DD-transpeptidases, also known as penicillin binding proteins (PBPs), that cross-link the pentapeptide side chains of adjacent glycan strands in the peptidoglycan (PG). The MICs for penicillin against enterococci are often one to two logs higher than those of closely related streptococci (232). The level of intrinsic resistance varies greatly by species, with *E. faecium* being generally more resistant than *E. faecalis* (234). Penicillins exhibit the most useful activity against enterococci followed by carbapenems, which lack activity against *E. faecium*. Last, enterococci are generally insensitive to cephalosporins at clinically achievable concentrations (232, 235).

Broadly, low to moderate levels of intrinsic resistance of enterococci to penicillins depend on the activity of low-affinity PBPs named Pbp5 in *E. faecium* and *E. hirae* and Pbp4 (occasionally known as Pbp5) in *E. faecalis*. Low-affinity PBPs are characterized by their weak binding to β-lactams, allowing them to carry out transpeptidase activity when the activity of other PBPs with higher β-lactam affinities are inhibited (Fig. 7). Initially described in *E. hirae*, Pbp5 is chromosomally encoded and was shown to have approximately 10-times-lower affinity for penicillin than other PBPs (236). Several lines of evidence point to a low-affinity PBP as a key determinant of penicillin tolerance in enterococci. First, closely related group A, B, and G streptococci lacking low-affinity PBPs are several hundred times more susceptible to penicillin than enterococci (236). Second, the MIC for penicillin corresponded to the concentration at which 90% of Pbp5 is saturated, well above the saturation point of any other PBP from *E. hirae* (236). Last, inactivation of *pbp5* in *E. faecium* resulted in hypersensitivity to penicillin (237). The same low-affinity PBP-mediated mechanism has been described in detail for both *E. faecalis* (238) and *E. faecium* (239–241). In *E. faecalis*, a strain isolated from a persistent prosthetic knee joint infection showing high MICs for penicillins and carbapenems was found to have two point mutations with amino acid substitutions (V223I and A617T) in Pbp4 that lowered Pbp4 affinity for β-lactams (A617T) while increasing protein expression (V223I) (242). Thus, the reduced susceptibility of this clinical isolate resulted from a combination of remodeling of the Pbp4 active site and increased protein expression. The intrinsic cephalosporin resistance of *E. faecalis* depends on several pathways involved in PG metabolism. Although not as extensively explored as penicillins, low-affinity PBPs contribute to intrinsic resistance against cephalosporins in *E. faecalis*. An isogenic mutant of *E. faecalis* JH2-2 lacking Pbp4 (named Pbp5 in the study) shows a

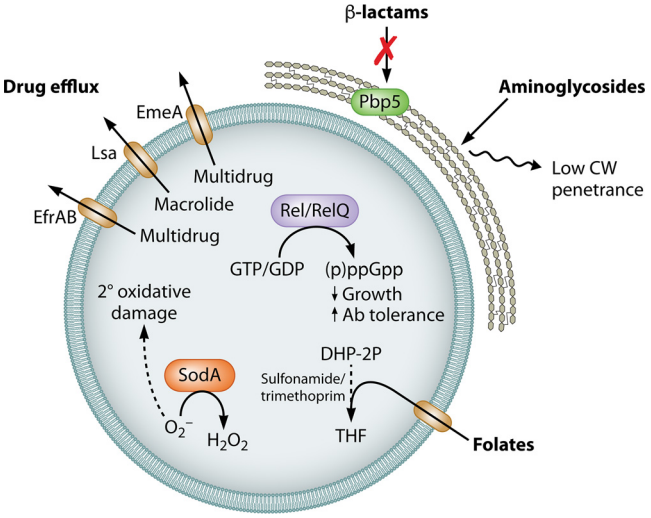

FIG 7 Intrinsic antibiotic resistance mechanisms. Enterococci are notoriously tolerant to the effects of clinically achievable concentrations of aminoglycosides, which stems from the low penetrance of this antibiotic class through the thick peptidoglycan layer. Intermediate tolerance to β-lactam antibiotics is, in part, the result of the action of the Pbp5, which displays 10-fold-lower susceptibility to these cell wall (CW) inhibitors than other enterococcal Pbps. Uptake of exogenous folate from the environment circumvents inhibition by the antibiotic cocktail sulfonamide and trimethoprim. The drug efflux pumps EfrAB, Lsa (*E. faecalis* specific), and EmeA are responsible for the extrusion of multiple antibiotic classes, reducing the intracellular levels of these drugs to subinhibitory or sublethal levels. General antibiotic tolerance in *E. faecalis*, and likely in other enterococci, is induced through activation of the stringent response and scavenging of the highly reactive superoxide molecule before it damages key cellular macromolecules. Stringent response induction and accumulation of the nucleotide second messenger (p)ppGpp reduce macromolecule biosynthesis and growth but also activate stress protective mechanisms, thereby reducing the number of active drug targets and enhancing antibiotic tolerance.

4,000-fold reduction in the MIC to ceftriaxone compared to that of the wild-type strain (238). The high saturation point of this low-affinity PBP for penicillin suggests that a similar mechanism contributes to an intrinsically high ceftriaxone MIC in *E. faecalis*.

The eukaryotic-type Ser/Thr kinase IreK, conserved among enterococci, is critical for broad-spectrum cephalosporin resistance, with the *ireK* mutant strain of *E. faecalis* OG1RF showing over a 100-fold reduction in MIC (243). MurAA is one known target of IreK that has been shown to enhance intrinsic cephalosporin resistance. MurAA is a UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase catalyzing the phosphoenolpyruvate (PEP)-dependent conversion of UDP-GlcNAc to UDP-GlcNAc-EP (244), the first committed step in PG synthesis. Overexpression of MurAA complements the cephalosporin resistance defect of an *ireK*-deficient strain, indicating that MurAA functions downstream of IreK (244). However, a lack of change in the endogenous *murAA* expression levels between wild-type and Δ*ireK* mutants of *E. faecalis* indicates that IreK controls MurAA enzymatic activity but not transcription (244). How MurAA catalytic activity enhances cephalosporin resistance, the mechanism by which IreK regulates MurAA activity, and the signal(s) sensed by IreK is still an open question.

In addition to IreK, a second, highly conserved signal transduction system, CroRS, also contributes to intrinsic cephalosporin resistance (245, 246). Deletion of *croRS* reduces the MIC for ceftriaxone in *E. faecalis* JH2-2, similar to the effect of the *pbp4* mutant albeit it did not affect the expression of *pbp4* (238, 246). Few genes are known to be regulated by CroRS, and none of these have an apparent role in cephalosporin resistance (247, 248). However, the diverse signals and interactions governing the activity of the CroRS are becoming better defined. A panel of cell wall-inhibiting antibiotics, targeting a range of steps involved in the synthesis and assembly of PG but no other cellular processes, induces expression of the *croRS* operon (246). Thus, it seems that CroRS monitors some aspect of PG synthesis to promote cephalosporin resistance. In addition, exogenous or endogenous H_2O_2 can induce *croRS* expression, resulting in

enhanced cephalosporin resistance. As CroS, the predicted sensor histidine kinase, lacks any known redox sensing motif, activation by H_2O_2 is likely an indirect result from oxidative damage to PG biogenesis (249). Finally, the predicted response regulator (RR) CroR interacts with the PTS regulatory protein HPr. This interaction blocks the ability of CroR to stimulate cephalosporin resistance in *E. faecalis* (250). Despite this progress in the understanding of the CroRS pathway, the signal or signals sensed by CroRS in response to perturbation of PG synthesis and the downstream pathways controlled by CroRS are still elusive.

Aminoglycosides. Aminoglycosides are a class of antibiotic that bind at the acceptor site (A-site) of the small ribosomal subunit to cause codon misreading and inhibition of tRNA translocation to the peptidyl-tRNA site (P-site). As a result, aminoglycosides block protein synthesis and lead to higher translational error rates and the subsequent accumulation of toxic proteins thought to be responsible for their bactericidal effects (251–253). Enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides; this intrinsic resistance is attributed to the low penetrance of aminoglycosides into the cell (Fig. 7). Treatment of *E. faecalis* with antibiotics interfering with PG synthesis, but not compounds targeting protein or membrane biogenesis, leads to enhanced uptake of aminoglycosides and a synergistic killing effect (254, 255). This implicates the enterococcal cell wall as the barrier to aminoglycoside uptake and intermediate levels of resistance. Furthermore, ribosomes isolated from *E. faecalis* exhibiting intermediate aminoglycoside resistance are inhibited by streptomycin, thereby confirming that target modification is not at play and further supporting the idea that resistance results from failure of aminoglycoside to reach the ribosome (256). Additionally, the emergence and rapid spread among enterococci of a gene encoding a large bifunctional enzyme that mediates high-level resistance to nearly all aminoglycosides (257) coincide closely with the emergence of endemic pathogenic hospital lineages (17).

Sulfamethoxazole-trimethoprim. Tetrahydrofolate is an essential coenzyme participating in one-carbon exchanges required for the synthesis of purine nucleotides, methionine, and thymine. Sulfonamides, including sulfamethoxazole, are competitive inhibitors of dihydropteroate synthetase, and trimethoprim is an inhibitor of dihydrofolate reductase. The combination of these two drugs has a synergistic effect, blocking adjacent steps in the *de novo* tetrahydrofolate biosynthesis pathway. However, sulfamethoxazole-trimethoprim is ineffective at treating enterococcal endocarditis in the clinical setting (258) and in animal models (259, 260). This lack of *in vivo* efficacy is attributed to the ability of enterococci to take up exogenous folate from the environment (Fig. 7) (258, 261).

Multidrug resistance efflux pumps. Drug efflux pumps are transmembrane proteins that expel toxic compounds from the cytoplasm and periplasm of the bacterial cell, preventing their ability to inhibit vital physiological processes. These efflux pumps utilize either the proton motive force or ATP, in the case of ABC family transporters, to drive drug extrusion. Both *E. faecalis* and *E. faecium* actively efflux numerous antibiotics, including norfloxacin, chloramphenicol, tetracycline, and benzylpenicillin (262), while treatment of *E. faecalis* with efflux pump inhibitors can block the export of norfloxacin (263).

Three major chromosomally encoded efflux pumps have been characterized in enterococci (Fig. 7). EmeA, for enterococcal multidrug resistance efflux, was identified as a homolog of NorA, the fluoroquinolone resistance protein and efflux pump from *S. aureus* (263, 264). Inactivation of *emeA* contributed to a modest but consistent reduction in resistance to fluoroquinolones, lincosamides, macrolides, and the aminocoumarin antibiotic novobiocin. Although efflux was not directly assessed for these antibiotics, EmeA actively exports ethidium bromide, enhancing resistance to this DNA-intercalating agent (263). EfrAB is a second multidrug efflux pump in *E. faecalis* that appears to constitute an ATP-dependent ABC family transporter and is conserved among many *Enterococcus* spp. with greater than 80% amino acid sequence identity (265). Expression of EfrAB in *E. coli* enhanced resistance to fluoroquinolones, doxycy-

cline, and a synthetic aminoglycoside, indicating that EfrAB may function similarly in *E. faecalis* (265). A second ABC transporter, known as Lsa, is responsible for the species-specific resistance of *E. faecalis* to the streptogramin combination quinupristin-dalfopristin and the lincosamide clindamycin (266). Streptogramins are a class of protein synthesis inhibitors, related to macrolides and lincosamides, which bind the peptidyl-transferase domain of the large ribosomal subunit to block translation. Similarly, lincosamides bind to the 50S rRNA of the large ribosomal subunit to inhibit translation. Deletion of Lsa causes a large reduction in quinupristin-dalfopristin and clindamycin MIC values. The efflux properties of Lsa have not been assessed directly, but transfer of Lsa from *E. faecalis* to *E. faecium* conferred a significant increase in MIC for quinupristin-dalfopristin (266). While drug efflux pumps are important mechanisms contributing to the intrinsic low-level resistance of enterococci to numerous antibiotic classes, the number and specificity of these pumps are likely to vary by species and, therefore, so will their relative contributions to intrinsic antibiotic resistance.

Antibiotic tolerance. The phenomenon of antibiotic tolerance was first noted in enterococci with the observation that very high doses of penicillin, many times above typical MIC values, are needed to kill >99.9% of *E. faecalis* cells (267, 268). This apparent lack of bactericidal activity was attributed to the presence of persister cells (268). Persister cells are thought to represent a small subpopulation of metabolically inactive and nondividing cells. Since most antibiotics require active growth to corrupt their target biological process, persister cells are inherently multidrug tolerant. Persistence is also distinct from antibiotic resistance as it is not a heritable trait. The offspring of persisters remain sensitive to antibiotics upon reactivation once the stress is removed. The initiation and maintenance of persistence are still not fully understood but appear to be complex, involving multiple initiation signals and functionally redundant mechanisms to keep cells in a dormant state (269). However, the regulatory nucleotide (p)ppGpp has emerged as a central determinant in this process (270–274). The persistence activation cascade has not been well defined in Gram-positive bacteria, including enterococci, but there is positive correlation between (p)ppGpp levels and antibiotic tolerance in *E. faecalis* (Fig. 7) (209, 211, 214).

Multidrug tolerance in *E. faecalis* has also been linked to the oxidant-scavenging activity of SodA (Fig. 7). Abrogation of SodA activity broadly sensitizes several strains of *E. faecalis* to lethal effects of numerous antibiotic classes (275, 276). Furthermore, levels of *sodA* expression directly correlate with vancomycin tolerance levels in *E. faecalis* and most *E. faecium* strains tested (276). In *E. faecium* HM1070, there was no direct correlation between *sodA* expression and vancomycin tolerance. However, SodA was critical for resisting killing by penicillin, suggesting that *E. faecium* HM1070 is unique and that its sensitivity to vancomycin tolerance stems from an intrinsic cell wall defect (276). In addition, a link between (p)ppGpp, activation of antioxidant pathways, and enhanced antibiotic tolerance has been observed in both *E. faecalis* (211) and the distantly related *Pseudomonas aeruginosa* (272, 274), suggesting that reduction in superoxide levels may also assist in the maintenance of persistence.

Summary and perspectives. Antibiotics have been increasingly applied to ecologies such as those of hospitals and agricultural settings proximal to humans (277, 278) and are no longer limited to isolated locations in soil where they are naturally produced. Enterococci are armed with a diverse set of mechanisms making them inherently resistant to several classes of antibiotics. This intrinsic resistance likely places them in ecologies in close contact with microbes that achieved resistance by other mechanisms, including resistances conferred by mobile elements. It may be possible to use our current understanding of the molecular mechanisms mediating antibiotic resistance and tolerance to develop adjuvant compounds to target this process and enhance antibiotic susceptibility. Efflux pump inhibitors are currently under development in addition to use of less traditional approaches such as the (p)ppGpp synthetase inhibitor relacin that could potentially reduce levels of antibiotic resistance and tolerance (279, 280). Inhibitors of folate uptake are also attractive targets for drug development as they would make enterococci susceptible to trimethoprim-sulfamethoxazole *in vivo*. Similar

strategies are being considered to extend the useful lifetime of folate synthesis inhibitors for the treatment of malaria caused by the parasite *Plasmodium falciparum* (281).

Disinfectants

While enterococci are generally susceptible to hospital-grade disinfectants (282–284), reduced chlorhexidine susceptibility in enterococci isolated from intensive care unit (ICU) patients has been reported (285), and the emergence of *E. faecium* subpopulations with reduced chlorhexidine susceptibility after exposure to subinhibitory concentrations of this antimicrobial has been demonstrated in the laboratory setting (286). Chlorhexidine is a broad-spectrum cationic disinfectant commonly used as an antiseptic, including preoperative surgical scrubs and whole-body rinsing of ICU patients to control vancomycin-resistant enterococci (VRE) and other nosocomial infections. A global transcriptional analysis of *E. faecalis* V583 exposed to subinhibitory concentrations of chlorhexidine identified an ABC-type transporter (EfrE-EfrF) and the adjacent MerR-like transcriptional regulator (named ChlR) as key players of the *E. faecalis* chlorhexidine stress response (287). Moreover, a transposon mutant library constructed in a multidrug-resistant *E. faecium* bloodstream isolate (E1162 strain) identified a putative two-component system (named ChtRS) that contributed to *E. faecium* chlorhexidine tolerance (288). In addition to chlorhexidine, few studies have identified traits that confer enterococcal tolerance to other commonly used hospital disinfectants. In one study, a small multidrug resistance transporter homologous to staphylococci *qac* genes was identified in approximately 60% of clinical isolates of *E. faecalis* and *E. faecium* (289). The protein encoded by the *qac* gene, termed QacZ, was shown to increase *E. faecalis* tolerance toward benzalkonium chloride, a surfactant quaternary ammonium compound used as an antimicrobial agent in soaps, hand wipes, creams, and household cleaning products. In another study, it was found that contemporary strains of *E. faecium* isolated after 2010 were about 10 times more tolerant to killing by isopropanol used in alcohol-based hand disinfectants than strains isolated between 1997 and 2010 (290). These alcohol-tolerant strains accumulated mutations in genes involved in carbohydrate uptake and metabolism, particularly PTS genes (290).

Host-Derived Stresses

Enterococci colonizing the animal GI tract must contend with numerous host defenses. Illustrating the innate ruggedness of enterococci, they are able to withstand the mechanical and enzymatic forces in the grinder of the bacterium-eating nematode, *C. elegans* (291). Moreover, both *E. faecalis* and *E. faecium* are capable of proliferating in the *C. elegans* intestine, despite innate immune and digestive factors (291). It is possible that surviving predation by early forms of Bilateria and accumulation in the animal intestine led to the first formation of cooperative GI tract consortia, the origin of the first gut microbiota (4). This likely involved selection for the ability to survive mechanical disruption, antimicrobial defenses, and the hydrolytic properties of host digestive systems, preparing enterococci to become successful human commensals. The corresponding bacterial responses to these host-derived stresses are complex, integrating a number of the stress adaptation mechanisms already described above.

AMPs. To colonize a diverse array of hosts and body sites, enterococci must withstand multiple host innate defenses. A major component of the host innate immune system is the production of antimicrobial peptides (AMPs). AMPs are produced at high levels by host cells such as the epithelia and phagocytes that commonly interact with microbes. These AMPs can be found at numerous body sites, including the skin, oral cavity, GI tract, respiratory tract, and the eyes (2, 3, 292–294). Smaller-molecular-weight AMPs typically exert their antibacterial properties through nonenzymatic means by disrupting the cytoplasmic membrane (2). Large AMPs, such as lysozyme, commonly have lytic functions, bind critical metabolites, or bind specific targets on microbial cells (2).

As a genus, *Enterococcus* displays remarkable resistance to lysozyme and is almost

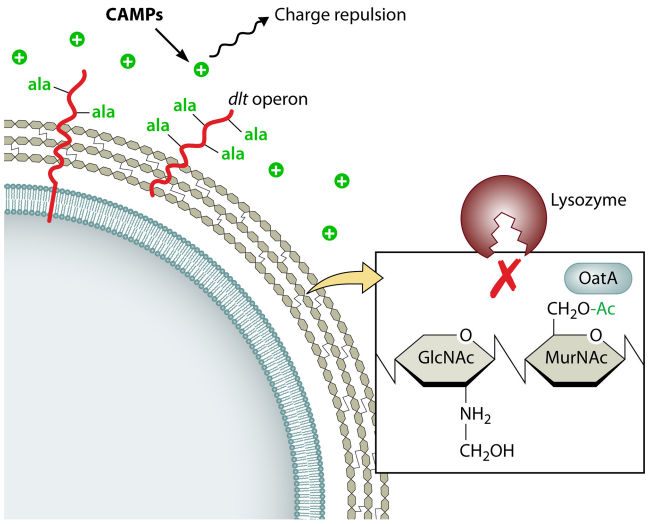

FIG 8 Resistance to antimicrobial peptides. The O-acetylation of *N*-acetylmuramic is a key modification of the peptidoglycan, reducing the affinity of lysozyme for its substrate, the *N*-acetylglucosamine *N*-acetylmuramic acid dimer, contributing to the intrinsic lysozyme resistance of enterococci. More generally, the alanylation of lipoteichoic acid (LTA) and teichoic acid (TA) by the *dlt* operon creates greater positive charge on the cell surface, preventing the association of small nonenzymatic CAMPs but also enzymatically active CAMPs like lysozyme.

completely recalcitrant to its antimicrobial effects (295, 296). The antimicrobial activity of lysozyme is due to cleavage of the β-1,4 linkage between *N*-acetylmuramic acid (NAM) and *N*-acetyl-D-glucosamine (NAG) residues in the PG that weakens the bacteria cell wall (297). In *E. faecalis*, three separate and functionally redundant cell wall modifications are known to contribute to lysozyme tolerance: O-acetylation of NAM carried out by OatA, deacetylation of NAG by PgdA, and alanylation of teichoic acids (TA) and lipoteichoic acids (LTA) by the *dlt* operon (Fig. 8). Alteration of PG structure by OatA and PgdA reduces substrate affinity or causes steric hindrance protecting cells from lysozyme activity (297). Alanylation of TA and LTA reduces the net negative charge of the cell surface, lowering the binding affinity of positively charged lysozyme and other positively charged AMPs (298).

In initial studies, deletion of *oatA*, but not *pgdA*, was shown to have a detrimental impact on lysozyme resistance in the JH2-2 strain when cells were grown on solid medium containing over 10 mg/ml lysozyme (299). In comparison, wild-type cells are resistant to lysozyme concentrations up to 50 mg/ml (299). Although PgdA did not substantially enhance lysozyme tolerance, *in vitro* experiments confirm that PgdA possesses deacylase activity and is transcriptionally induced by lysozyme (300). Deletion of *dltA*, eliminating the ability of *E. faecalis* to alanylate LTA, results in similar levels of lysozyme sensitivity to those observed in the *oatA* deletion strain (299, 301, 302). However, it should be noted that strain-to-strain differences appear to strongly influence the relative contribution of cell wall modification mechanisms to lysozyme tolerance. Phenotypical analysis of *oatA* and *dltA* mutants created in JH2 and V583 strain backgrounds of *E. faecalis* did not significantly impact lysozyme resistance (303).

The regulation of stress responses that combat cell envelope stresses, including AMPs, is often orchestrated by a class of alternative extracytoplasmic sigma factors (ECFs) (304). The ECF sigma V (SigV) of *E. faecalis* JH2-2 is activated following lysozyme treatment and strongly contributes to lysozyme tolerance among other cell envelope stresses, including ethanol, acid, and heat (305). To ensure proper control of ECF activity, a transmembrane anti-sigma protein binds and inhibits the ECF until the correct cell wall stress stimulus is provided, at which time the anti-sigma factor is inactivated by extracellular and cytoplasmic proteases (304, 306). Similarly, the activity of SigV was shown to be under dual-layer control by the anti-sigma RsiV and the

membrane-bound metalloprotease Eep (303, 305). The proteolytic events upstream of Eep that initiate the cellular response to lysozyme are yet to be identified. A combination of *in silico* and transcriptional analysis shows that SigV activates *pgdA* and itself, explaining the strong induction of both genes in the presence of lysozyme (300, 302). Regulation of *oatA* and the *dlt* operon appears to be independent of SigV (302). However, transcriptional induction of *pgdA* by SigV does not fully account for the contribution of SigV to lysozyme resistance as deletion of *pgdA* had no significant impact on lysozyme resistance (299). From this, it appears that SigV contributes to lysozyme resistance through additional mechanisms.

In addition to lysozyme resistance, D-alanine modification of LTAs by the five-gene *dlt* operon (*dltABCD* and *dltX*) in *E. faecalis* leads to the production of amphipathic, membrane-anchored TA that have a broader protective role against cationic AMPs (CAMPs) (Fig. 8). CAMPs are attracted to the anionic components on the surface of bacterial cells like lipids or TA, driving nonspecific association with the cell surface followed by penetration of the membrane by the amphipathic portion (3). The *dltABCD* operon is responsible for the two-step process for esterification of D-alanine to LTA. Deletion of any gene of the *dlt* operon is sufficient to abolish LTA D-alanylation due to the sequential nature of the pathway. The deletion of *dltA* significantly impairs resistance to different antimicrobial peptides such as colistin, polymyxin B, and nisin (307). This reduced resistance is most likely the result of an increase in the overall negative charge of the cell surface and enhanced electrostatic attraction to CAMPs, as seen in *S. aureus dlt* operon mutants (298). Another mechanism utilized by *E. faecalis* to resist CAMP killing is based on the activity of the well-studied multiple peptide resistance factor 2 (MprF2), a membrane-associated protein that aminoacylates the PG by adding cationic peptides to phospholipids, thereby conferring CAMP resistance by electrostatic repulsion (308, 309). As seen in other Gram-positive bacteria, inactivation of *mprF2* increased susceptibility against several CAMPs *in vitro* albeit loss of MprF2 did not affect neutrophil survival or virulence in a mouse bacteremia model (309). Given that production of antimicrobial peptides is a conserved mechanism used by animals ranging from nematodes to humans to control the GI microbial community, the modulation of surface charge in enterococci is likely to be a critical attribute for their ability to stably colonize and survive within their natural habitat.

Regulation of the *dlt* operon in *E. faecalis* appears to be under multilayer control that involves several two-component regulatory systems (TCS). Inactivation of the response regulators (RR) RR03 and RR13 from their corresponding TCSs increases *dlt* expression while inactivation of RR09 causes a slight reduction in *dlt* expression during logarithmic growth (310). RR13 belongs to the OmpR regulatory family and shares a high level of homology with the SrrA transcriptional regulator from *S. aureus*. SrrA is responsible for the repression of virulence genes under low-oxygen conditions (311). RR09, also a member of the OmpR family, bares little homology to any described response regulators (245, 312), and the signals sensed by this TCS are not yet known. Deletion of *ireK* negatively impacts stress phenotypes associated with cell envelope stress (243), and it has been suggested that the *dlt* operon could be under its control (310). In *E. faecium*, the *dlt* operon is negatively regulated by AsrR (antibiotic and stress responsive regulator), a redox-sensing transcriptional regulator. Deletion of *asrR* increased expression of the *dlt* operon and enhanced resistance to nisin (313). AsrR homologs can be identified in numerous enterococcal species, including *E. durans*, *E. hirae*, *E. gallinarum*, and *E. casseliflavus*, but are absent in *E. faecalis*.

Bile. Bile is an essential digestive fluid secreted into the GI tract that emulsifies and solubilizes fat from the host diet. The major components of bile include bile acids, phospholipids, cholesterol, and the pigments biliverdin and bilirubin (314). In addition to its function as a biological detergent, bile possesses strong antimicrobial activities and therefore plays an important role in the host biochemical defense system. Membrane damage appears to be the primary antimicrobial mechanism of bile as alteration of membrane integrity through mutation enhances bile sensitivity (314). Furthermore, adaptation to physiochemical stresses that alter membrane characteristics, including

acid and osmotic stresses and entry into stationary phase, enhances bile tolerance (31, 314). Outside the cell membrane, bile may have secondary effects on other critical cell processes that include alteration to nucleic acid secondary structure, ROS generation, cytoplasmic dissociation of bile salts causing intracellular acidification, and the chelation of intracellular iron and calcium (314). As a result of these numerous secondary effects, the activation of corresponding adaptation pathways is observed, including antioxidant responses, protein chaperones, and ATP-driven proton pumps (314).

Exposure of *E. faecalis* ATCC 19433 to sublethal concentrations of bile salts for 30 min can confer increased protection to a subsequent challenge with lethal concentrations (315). Remarkably, exposure times as short as 5 s can enhance survival by over 1 log (315). Early proteomic analysis of cells treated with bile identified 45 proteins with increased cellular abundances (315). Of these proteins, the protein chaperones GroEL and DnaK were positively identified (31). As mentioned previously, many of these proteins also accumulated in response to numerous other stresses (10, 12). Two of these so-called general stress proteins, Gls24 and GlsB, were shown to directly contribute to bile tolerance as inactivation of both Gls24 and GlsB reduced survival in bile by more than 90% (316). Homologous *gls* genes in *E. faecium* also contribute to survival in bile (317). The mechanisms by which these general stress proteins contribute to bile salt resistance are currently unknown.

A more contemporary proteomic analysis identified 53 unique proteins differentially regulated in response to bile in *E. faecalis* V583 (318). This profiling confirmed earlier findings that protein chaperones are a major component of the bile stress response but also found a selective reduction in the levels of proteins involved in fatty acid biosynthesis (318). Alterations of the membrane fatty acid composition are known to affect the biophysical properties of the membrane, the primary target of bile. In fact, *E. faecalis* OG1RF is able to incorporate large amounts of exogenously added long-chain fatty acids ($\geq$16 carbons), up to 90% of their total fatty acid profile, dramatically modifying their membrane content (319). Exogenous addition of the host-derived oleic acid (18:1 *cis*-9) protects against lethal challenge with bovine bile, resulting in a 2-log increase viability. Oleic acid is also present in serum and bile, and growth of *E. faecalis* supplemented with either serum or bile increases the membrane content of oleic acid and enhances bile resistance (319). The reduction of fatty acid biosynthesis proteins seen in bile-grown cells could then represent feedback repression of the type II fatty acid biosynthesis pathway resulting from the incorporation of exogenous fatty acid from bile.

Transcriptional analysis found a similar strong repression of the fatty acid biosynthesis pathway but also uncovered the activation of two pathways involved in maintenance of proton homeostasis: the previously mentioned ADI pathway and the V-type sodium exporting ATPase needed for generation of an electrochemical gradient under alkaline pH (320). Activation of the ADI pathway may reflect an attempt to counter cytoplasmic acidification by bile through generation of ammonia, while increased expression of the V-type ATPase may boost the electrochemical gradient to maintain the proton motif force during cytoplasmic acidification. By employing an insertional mutagenesis approach, Breton and coworkers identified 10 loci important for bile tolerance corresponding to diverse cellular functions that included fatty acid biosynthesis, DNA repair, pyruvate oxidoreductase (interconverts pyruvate and acetyl-coenzyme A [CoA]), and cell wall metabolism (321). Finally, the EpaX glycosyltransferase located in the variable region of the *epa* locus of *E. faecalis* V583 was shown to promote resistance to bile salts and to facilitate gut colonization by decorating the rhamnose-based precursor of Epa with galactose and/or *N*-acetyl galactosamine (GalNAc) (322).

Regulation of adaptation to bile is not well understood in other bacteria but is thought to involve the general stress sigma factors σ^B and σ^S in *L. monocytogenes* and *E. coli*, respectively (314). However, the genomes of enterococci lack both σ^B and σ^S factors. A transcription factor, *slyA*, initially characterized for its contribution to virulence, enhances *E. faecalis* growth in bile (323). Global transcriptional profiling indicated that SlyA controls the expression of over 100 genes, primarily by functioning as a

transcriptional repressor (324). While the vast majority of these genes do not appear to function in pathways known to contribute to bile stress tolerance, SlyA activates the expression of two putative cholylglycine hydrolases that metabolize conjugated bile acids in bacteria (323). In addition, the serine/threonine kinase IreK, crucial for cephalosporin resistance in *E. faecalis* as described earlier, also contributes to bile tolerance (243). Intestinal colonization by the *ireK* mutant was 6-fold lower than that of the parent, drawing a possible correlation between bile tolerance and GI colonization and persistence (243). Notably, the genomes of numerous sequenced *Enterococcus* species contain a Mar family transcriptional regulator and serine/threonine kinase that share strong amino acid sequence homology to SlyA and IreK, respectively. Therefore, it is likely that these regulators contribute more broadly to control bile adaptation across enterococci.

Urine. Enterococci are a leading cause of chronic and recurrent hospital-associated urinary tract infection, particularly in intensive care units, and are most commonly associated with patients having structural abnormalities or instrumentation, including catheterization (232, 325). The prominence of enterococci in urinary tract infection indicates that they are well equipped to grow and survive in urine. Although urine is able to support the growth of numerous bacterial pathogens including enterococcal species, it does impose nutritional and osmotic stresses (326, 327). Human urine is rich in nitrogen due to the high levels of urea (>100 mM), phosphorus, and potassium but is generally limiting for amino acids, particularly BCAAs, and micronutrients like free iron that is sequestered by lactoferrin (327–329). Urine also has low, submillimolar concentrations of glucose, the preferred sugar substrate of enterococci (327, 328). Bacterial growth and survival in urine are further complicated by constant fluctuations in osmolarity as the body responds to temporary water imbalances and the presence of host AMPs like α- and β-defensins and cathelicidin (330).

The transcriptional response of three *E. faecalis* strains following growth in human urine has been examined by microarrays and indicates that broad physiological alterations are necessary to adapt to this environment (331). Transcriptional profiles show signatures of alleviation of carbon catabolite repression (CCR), which is in line with the normally very low glucose levels detected in urine (327, 328, 331). This includes repression of the mannose PTS, a major glucose/mannose uptake system, and activation of citrate metabolism and alternate sugar transporters (328). A shift toward citrate metabolism may constitute an important metabolic adaptation as citrate was shown to be present at low millimolar concentrations in urine (327). As urine is also limiting for numerous amino acids, many of which are required to support *E. faecalis* growth, it is not surprising that transcription of peptide and amino acid transport systems and of amino acid biosynthesis genes is induced in urine, while amino acid catabolic genes are repressed (327, 331). There is also an activation of genes involved in Mn and Fe uptake, supporting evidence that urine is limiting for these essential micronutrients (331, 332). Together, these transcriptional alterations indicate that urine poses a strong nutritional stress but one that can be overcome through the activation of appropriate transport and biosynthesis pathways.

Recombinase *in vivo* expression technology (RIVET) has also been used to identify genes important for growth and survival in urine (333). The RIVET screen confirms several patterns of transcriptional alteration from the microarray studies, as several promoters of genes involved in nutrient transport are activated during growth in urine (333). Activation of the promoter regions coding for the two subunits of oxaloacetate decarboxylase, which converts oxaloacetate to pyruvate as part of the citrate metabolism pathway, further supports the importance of citrate metabolism to *E. faecalis* growth in urine (333). Since global nutritional regulators like CcpA, CodY, and (p)ppGpp are closely tied to the regulation of many of these transport and metabolic pathways identified by microarray and RIVET, it seems likely that they might serve as important sensors coordinating the transcriptional changes observed following growth in urine.

Blood. Human blood is rich in carbohydrates and peptides but poor in free amino acids and essential biometals (334). In the United States, enterococci are a leading

cause of bloodstream infections in intensive care units, second only to coagulase-negative staphylococci (325). Thus, despite their numerous amino acid auxotrophies (212), enterococci can adapt, grow, and survive in blood. Global transcriptional profiling of *E. faecalis* grown in whole blood or in medium supplemented with 10% blood was performed to identify the molecular mechanisms involved in this adaptive process (169). Much like cells grown in urine, cells grown in blood show a CCR alleviation signature. This includes the downregulation of major glucose PTS genes and genes participating in the initial steps of glycolysis and activation of genes involved in secondary carbon metabolism (169). Unexpectedly, these transcriptional changes suggest that glucose, ranging from 4 to 8 mM in most individuals, is limiting in blood but that *E. faecalis* can compensate through transport and utilization of alternative sugar and carbon sources. Also, as seen in urine, transporters for both iron and manganese were upregulated, as would be suspected since iron is tightly complexed with iron binding proteins and since serum manganese levels have been reported to be in the nanogram/milliliter range (169, 190). As blood is limiting for free amino acids, a number of oligopeptide transporters are activated, suggesting that *E. faecalis* can supplement its amino acid requirements through the transport and utilization of small peptides from blood. Again, as many of these metabolic alterations are coordinated by the nutrient-sensing regulators (p)ppGpp and CodY, it is not surprising that both regulators are involved in the ability of *E. faecalis* to grow in whole blood (197). In addition to rerouting cellular metabolism, several stress responses, particularly antioxidant genes, appear to be important for growth and survival in blood. Specifically, the activation of *dps, sodA*, *npr*, *msrA*, and *msrB* suggests that blood exerts considerable oxidative stress on *E. faecalis* (169).

Summary and perspectives. Exposure to host-derived AMPs and exposure to bile are undoubtedly strong selective pressures to which enterococci were forced to adapt during their evolution into highly competitive members of the animal GI tract. Targeted genetic approaches have uncovered the importance of modulating the structural properties of PG, TA, and LTA to block the binding and lytic activity of host AMPs. However, these contributions may be overshadowed by SigV regulation, the regulon of which remains poorly defined. Transcriptional profiling experiments comparing an isogenic Δ*sigV* mutant with its wild-type counterpart have tremendous potential to reveal novel or yet to be appreciated mechanisms that contribute to the insensitivity of enterococci to lysozyme. Tolerance to bile in *E. faecalis* appears to be multifactorial, involving the activity of general stress proteins, acid tolerance response genes, and significant changes to the composition of the cell membrane. These diverse responses reflect the fact that bile not only targets the cell membrane through its detergent properties but also disrupts macromolecule function and electrochemical gradients through dissociation of bile salts. However, these bile tolerance pathways appear to constitute only a fraction of the underlying bile tolerance mechanism. The transcriptional regulators SlyA and IreK enhance bile tolerance, but their regulons are functionally perplexing and undefined, respectively.

Enterococcal bloodstream or urinary tract infections represent an evolutionary stalemate. Because the chance of blood-to-blood or urine-to-urine transmission is low, there is little advantage to these adaptive traits in sustained focal-site infection. Rather, the biochemical pathways for alternative carbon metabolism, peptide and amino acid transport, and uptake of Fe and Mn cofactors may be key to the success of enterococci in hosts of lower trophic levels with simpler, more restricted diets and to environmental survival during host-to-host transmission. Due to the limiting nature of blood and urine for certain key nutrients, these nutritional and metal homeostatic mechanisms have also proven beneficial during human infections.

CONCLUSIONS

We are now beginning to piece together the key genetic determinants and regulatory systems responsible for stress-specific and multistress tolerance in enterococci. It appears that these were selected initially as mechanisms for surviving predation and persistence in the environment and then, once the ability to colonize the host had

evolved, to survive repeated cycles of colonization and excretion. The ensuing ruggedness that evolved uniquely positioned the enterococci to persist in hospitals and agricultural settings and associate with other ecologically related microbes to acquire new resistances and virulence traits.

Some tolerance pathways, particularly those mediating adaptation to oxidative and pH stresses, are relatively well defined. Others, including osmotic and cold stress, remain poorly defined in both their molecular mechanisms and regulation. Advances in large-scale, data-rich biology such as transcriptomics have helped uncover numerous stress regulators and their corresponding regulons in enterococci. At present there are genetic tools for clean deletions, reporter systems, and random mutagenesis, several established animal models for studying urinary tract infection, endocarditis, and systemic infection, and fully sequenced and annotated genomes from numerous species. As a result, the enterococcal research community is now much better poised to dissect the interconnectivity of known stress response pathways and their regulators and also to identify novel survival mechanisms and investigate those less explored stress adaptation pathways mentioned above.

As the reader can note, most of the research presented here focuses on *E. faecalis*. Although *E. faecalis* continues to account for the majority of enterococcal infection in humans, the frequency of antibiotic resistance, particularly vancomycin resistance, among *E. faecium* nosocomial infections has greatly surpassed that of *E. faecalis* (335, 336). In the most recent report from the National Healthcare Safety Network, 83.8% of all *E. faecium* isolates were resistant to vancomycin in contrast to 9.9% of *E. faecalis* isolates (20). As a result, *E. faecium* clinical isolates are often multidrug resistant, further reducing the number of effective chemotherapeutic options and making *E. faecium* infections even more difficult to treat (337). Given the availability of fully sequenced genomes and tools for genetic manipulation of *E. faecium*, it would be important to examine in more detail stress adaptation in this underrepresented but medically important species. In addition, with the availability of sequenced genomes from numerous enterococcal species, a combination of comparative genomics and genetic analysis can provide clues as to how different species adapt to their unique ecological niches and also to what makes *E. faecalis* and *E. faecium* particularly well adapted to colonize the human host (338).

Although this review details our current understanding of both the effector proteins and regulatory elements mediating stress adaptation, a key question remains unanswered. What specific properties make enterococci more resilient than closely related commensals and pathogens from the lactic acid bacteria clade? Many, if not most, of the above adaptation mechanisms and regulatory elements detailed have genetic or functionally homologous counterparts within the *Lactobacillales*. Of the three best characterized enterococcus strains, *E. faecalis* V583 and OG1RF and *E. faecium* DO still have 28%, 22%, and 27% of their respective genomes comprised of genes with purely hypothetical functions. It is conceivable that there is a core set of uncharacterized hypothetical genes in enterococci that contribute to the intrinsic durability of the genus. To this end, recent comparative genomics efforts identified a core set of 1,037 genes, occurring in $>$90% of species, common to the genus *Enterococcus* (339). Of these, 126 core genes, one-third of which are hypothetical, were gained by enterococci since their divergence from their last common ancestor, the vagococcus, a common inhabitant of the GI tract of fish. These genes were functionally enriched for purine biosynthesis, cell wall modification, including lipid and wall teichoic acid modification by the *epa* and *dlt* operons, and stress responses (339). The divergent lifestyles of vagococci (aquatic) and enterococci (terrestrial) suggest that these 126 genes are critical for the survival of enterococci under unique environmental stresses, namely desiccation and starvation, selecting for their emergence along with animal terrestrialization approximately 500 million years ago.

Enterococci are also adept at sampling genes through horizontal transfer, and mobile genetic elements are common in both commensal and clinical isolates (340). Although most commonly associated with acquired antibiotic resistance and virulence,

human infection represents an evolutionary dead end. This means that the propensity of enterococci to harbor mobile elements evolved for a different purpose and contributes to enterococcal persistence by other means (4). A well-documented example is the pheromone-responsive plasmids by which *uvr* genes enhance resistance to lethal challenge with UV light in *E. faecalis* (341, 342). However, the contribution of mobile elements to environmental or host-derived stresses aside from UV light is limited. In the case of the enterococcal pathogenicity island, most genes have annotated functions unrelated to antibiotic resistance or overt virulence but appear to contribute to stable host colonization and expanded metabolic capacity (343). These circulating mobile elements offer a wide array of adaptive traits that can be rapidly acquired and disseminated but have been grossly understudied in enterococci relative to their potential impact on stress tolerance.

Given the possible mixes of core, accessory, and mobile genes, dissecting their individual and combined contributions to enterococcal resilience is a challenging problem due to genetic buffering and the lack of information regarding the function of hypothetical or ambiguously annotated genes. In the case of genetic buffering, deletion of any single gene is unlikely to result in a major fitness cost because stress adaptation is most often mediated by several proteins with complementary or redundant biochemical functions. However, high-throughput approaches like transposon sequencing (Tn-Seq) have proven to be powerful for identifying previously uncharacterized stress genes and genetic interactions to deconvolute complex stress phenotypes (344). Tn-Seq can also provide a quantitative measure of the relative fitness contribution of each gene or genetic interaction (345) and has been successfully applied to *S. aureus* (346).

Furthermore, in model organisms like *B. subtilis*, research has made significant headway into mapping the interconnected nature of numerous regulatory networks, allowing transcription factors and the genes to be grouped into biologically meaningful regulatory modules (347–349). Similar transcriptional and bioinformatics approaches are being applied to understand the interconnectivity and flexibility of homeostatic modules responding to metal stress in *E. faecalis* (350). Transcriptional responses to copper stress show three major response modules: (i) copper specific and limited to the *cop* operon, (ii) energy generation, and (iii) DNA damage and metal stress. Many members of modules ii and iii are also activated under Fe and Zn excess, regulated by members of the ArgR, LysR, and Fnr/Crp protein families, and appear to be linked to secondary effects of metal stress (350). Highlighting the intrinsic malleability of *E. faecalis*, deletion of the *cop* operon, the main Cu-homeostatic regulatory mechanism in *E. faecalis*, induces a dramatic alteration to the transcriptional landscape of the cell during periods of copper excess. Without the capacity to activate the copper efflux and repress copper uptake, *E. faecalis* instead downregulates ABC sugar transport and activates molybdenum uptake (350). The function of this response is not fully understood but may represent an attempt to induce metabolic arrest, reducing levels of macromolecules susceptible to ROS, and to sequester excess copper into coordinated metabolic pathways like purine biosynthesis (163, 350).

As more detailed information about stress proteins and their regulators are uncovered in enterococci, further computational modeling of transcriptional network architecture could define not only the regulatory modules contributing to adaptation to a given stress but also how these modules are connected leading to the impressive general stress tolerance and cross-tolerance of enterococci. As the ability of enterococci to survive under inhospitable conditions is, arguably, the major contributor to their rapid rise as major nosocomial pathogens, this is an important problem that will continue to require great attention.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MMBR.00008-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We are very grateful to Michael Gilmore (Harvard Medical School, Departments of Ophthalmology and Microbiology and Immunobiology) for encouraging us to write this review and drawing on his foundational work in the field of enterococcal biology to provide insightful comments throughout the writing process.

Our research was supported by National Institutes of Health grants AI135158 and AI137446 to J.A.L. During the drafting of this review A.O.G. was supported by American Heart Association grant 17POST33410288.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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