A eukaryotic-type Ser/Thr kinase in *Enterococcus faecalis* mediates antimicrobial resistance and intestinal persistence

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Antibiotic-resistant enterococci are major causes of hospital-acquired infections. The emergence of *Enterococcus faecalis* as a significant nosocomial pathogen is a consequence of its inherent resistance to certain antibiotics and of its ability to survive and proliferate in the intestinal tract. Genetic determinants of *E. faecalis* conferring these properties are largely unknown. Here we show that PrkC, a one-component signaling protein containing a eukaryotic-type Ser/Thr kinase domain, modulates inherent antimicrobial resistance and intestinal persistence of *E. faecalis*. An *E. faecalis* mutant lacking PrkC grows at a wild-type rate in the absence of antimicrobial stress but exhibits enhanced sensitivity to cell-envelope-active compounds, including antibiotics that target cell-wall biogenesis and bile detergents. Consistent with its bile sensitivity, the mutant was also impaired at persistence in the intestine of mice. Thus, PrkC regulates key physiological processes in *E. faecalis* associated with its success as a nosocomial pathogen. The predicted domain architecture of PrkC comprises a cytoplasmic kinase domain separated by a transmembrane segment from extracellular domains thought to bind uncross-linked peptidoglycan, suggesting that PrkC is a transmembrane receptor that monitors the integrity of the *E. faecalis* cell wall and mediates adaptive responses to maintain cell-wall integrity. Given its role in modulating traits of *E. faecalis* important for its ability to cause nosocomial infections, we suggest that the one-component signaling protein PrkC represents an attractive target for the development of novel therapies to prevent infections by antibiotic-resistant enterococci.

cell-envelope stress | one-component system | signal transduction | cephalosporin resistance | bile resistance

Bacteria maximize their fitness by using signal transduction pathways that modulate cellular functions in response to fluctuating environmental cues. Until recently, the predominant means of prokaryotic signal transduction was considered to be the two-component regulatory system (1). Two-component systems regulate a vast array of biological functions via phosphotransfer between two proteins: a sensor histidine kinase and a response regulator. These protein components are built with modular domain architecture (1, 2). Highly conserved phosphotransfer domains are linked to variable, stimulus-specific sensory input domains (in the kinases) and output domains (in the response regulators). Despite the widespread use of two-component systems, recent comparative genomics analyses suggest that the majority of prokaryotic signal transduction systems consist of a single protein that contains both input and output domains, but no phosphotransfer domains typical of those found in traditional two-component systems. Such signaling proteins, known as one-component systems (OCSs), now appear to be the dominant prokaryotic signaling system (3). For example, the genome of *Enterococcus faecalis* V583 encodes only 17 two-component systems (4) but has recently been predicted to encode 158 OCSs [http://genomics.ornl.gov/mist (3)]. The majority of OCSs are predicted to be DNA-binding transcriptional regulatory proteins, but comparative genomics analyses revealed many other OCSs exhibiting nontraditional domain architectures (i.e., OCSs with non-DNA-binding output domains). Because many such nontraditional OCSs have not been studied experimentally, the mechanisms of signal transduction and biological functions of these nontraditional OCSs are poorly understood.

Enterococci are low-GC Gram-positive bacteria whose primary habitat is the gastrointestinal tract of a wide range of animals (5, 6). However, their association with humans is not always benign, because enterococci are among the three most common causes of hospital-acquired infections (7) and have been since 1986 (8). Emergence of *E. faecalis* as a significant nosocomial pathogen is, at least in part, a consequence of its inherently robust nature. Enterococci are well known to be particularly resistant to a variety of environmental stresses (9–11), properties that have been widely used as the basis of schemes to isolate or differentiate enterococci from other Gram-positive cocci. Thus, the genetic determinants conferring resistance to such stresses must be encoded in the core genome of *E. faecalis*. In the context of hospital-acquired enterococcal infections, the inherent resistance of *E. faecalis* to stresses caused by cell-envelope-active antimicrobials is especially important. For example, inherent resistance of *E. faecalis* to cell-envelope-active detergents present in the intestinal emulsifying agent, bile, likely facilitates enterococcal colonization of the intestine, an important first step that usually precedes dissemination to other sites and the onset of infection (reviewed in ref. 12). Furthermore, some broad-spectrum cephalosporin antibiotics, which inhibit peptidoglycan cross-linking by penicillin-binding proteins (PBPs), are active against many members of the intestinal flora. Inherent resistance of *E. faecalis* to such cephalosporins enables *E. faecalis* to multiply to abnormally high intestinal cell densities when the ecological balance of the normal flora is disrupted in patients undergoing cephalosporin therapy (13), a known risk factor for enterococcal infection (reviewed in ref. 14). Despite the importance of these cell-envelope stress resistance traits, little is known of the intrinsic genetic determinants that enable *E. faecalis* to respond to the stresses caused by cell-envelope-active antimicrobials or, more generally, to efficiently colonize the intestinal tract. We sought to identify proteins encoded by

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Abbreviations: eSTK, eukaryotic-type Ser/Thr kinase; MIC, minimal inhibitory concentration; OCS, one-component system; PBP, penicillin-binding protein; Em, erythromycin; BHI, brain–heart infusion.

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the core *E. faecalis* genome that mediate these adaptive responses. Here we describe PrkC, a one-component signaling protein containing a eukaryotic-like Ser/Thr kinase domain that modulates inherent resistance to cell-envelope-active antimicrobials and intestinal persistence of *E. faecalis*.

Results and Discussion

To identify new signal transduction systems in *E. faecalis* that might mediate intrinsic enterococcal resistance to cell-envelope-active antimicrobial compounds, we searched the sequenced *E. faecalis* V583 genome (15) for ORFs predicted to encode one-component signaling proteins with nontraditional combinations of previously described signal transduction domains. Domain library browse and search tools available at the SMART (http://smart.embl-heidelberg.de) and Pfam (www.sanger.ac.uk/Software/Pfam) databases were used to identify *E. faecalis* genes encoding a Ser/Thr kinase domain (S_TKc domain, SMART accession no. SM00220; or Pkinase domain, Pfam accession no. PF00069). This search yielded a single gene (TIGR locus EF3120 in the *E. faecalis* V583 genome; GenBank accession no. NP_816730), denoted *prkC* based on sequence similarity to its homolog in *Bacillus subtilis*, encoding a eukaryotic-type Ser/Thr kinase (eSTK) domain. The eSTK domain of *E. faecalis prkC* is predicted to belong to the eSTK family with a high degree of statistical significance (*E* value of 1.64×10^{-69}) and contains all of the highly conserved residues characteristic of this family, including those required for catalytic activity.

The eSTKs are unrelated at the level of primary sequence or structure to the widely studied histidine kinases comprising typical bacterial two-component signaling systems. Although eSTKs are now known to be widely distributed in bacteria, in most cases their functions remain poorly understood (16–18). *E. faecalis* PrkC exhibits a characteristic bipartite domain architecture (Fig. 1*A*) comprising the presumably cytoplasmic eSTK domain separated by a transmembrane segment from a series of five presumably extracellular repeats of the PASTA domain (*E* values of 9.5×10^{-15}, 2.4×10^{-12}, 9.4×10^{-14}, 1.1×10^{-12}, and 2.8×10^{-11} for PASTA domains 1–5, respectively). The function of PASTA domains is unknown, but they are also found in a subset of PBPs (extracellular enzymes that mediate covalent cross-linking of peptidoglycan polymers) (19). In this context, PASTA domains have been proposed to bind to the free D-Ala-D-Ala peptide termini found in uncross-linked peptidoglycan. This suggests that the PASTA domains might serve as extracellular input domains that regulate the activity of the cytoplasmic eSTK (output) domain of PrkC. BLAST searches revealed that homologs of PrkC are present in the genomes of nearly all members of the low-GC-content Gram-positive bacteria as well as some high-GC Gram-positive bacteria. Thus, *E. faecalis* PrkC is a member of a new class of OCS, defined by its characteristic domain architecture, that is prevalent in Gram-positive bacteria. Although a few studies have investigated the role of PrkC homologs in their respective hosts, the functions of these proteins appear to be species-specific, ranging from a role in development of competence, virulence, surface protein expression, or regulation of intracellular nucleotide pools in some streptococci (20–24) to sporulation, stationary phase survival, or control of cell shape in other bacterial species (25–27).

To probe the function of *E. faecalis* PrkC, we used a recently described allelic exchange system (28) to construct an in-frame deletion of *prkC* in the chromosome of *E. faecalis* OG1RF, creating the Δ*prkC2* mutant CK119. Routine surveys of growth kinetics in different culture media revealed that the Δ*prkC2* mutant exhibited a doubling time nearly identical to its congenic wild-type parent in nutrient-rich brain–heart infusion (BHI) (Fig. 1*B*) and semidefined MM9YEG medium (data not shown), indicating the absence of a general growth

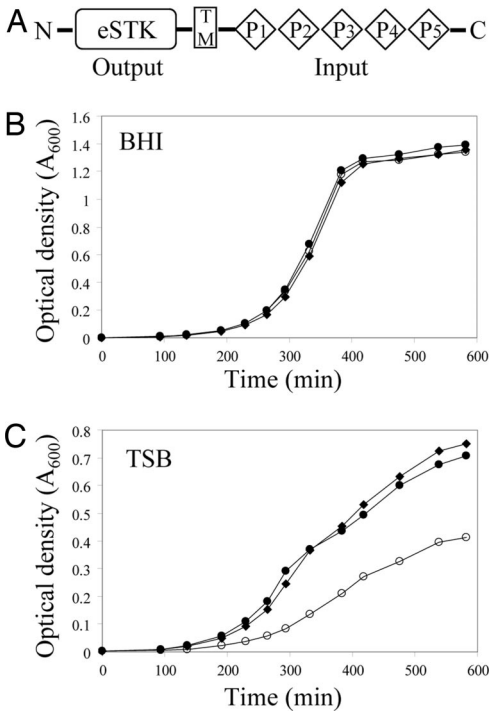

Fig. 1. Architecture of PrkC and growth properties of the Δ*prkC2* mutant. (*A*) Domain architecture of *E. faecalis* PrkC. TM, transmembrane domain; P, PASTA domain. Putative input and output signal transduction domains are indicated. (*B* and *C*) Analysis of growth kinetics in BHI (*B*) or tryptic soy broth (*C*) supplemented with 10 μg/ml Em (for plasmid maintenance) and 2% rhamnose (for induction of *prkC*). Representative growth curves are shown. The experiments were independently repeated three times with similar results. Diamonds, wild type [*E. faecalis* OG1RF(vector)]; open circles, Δ*prkC2* mutant [*E. faecalis* CK119(vector)]; filled circles, Δ*prkC2* mutant expressing *prkC* in trans [*E. faecalis* CK119(pCJK104)]. Vector was pCJK96.

defect of the mutant. The Δ*prkC2* mutant did, however, exhibit a growth defect when cultured in tryptic soy broth, which could be complemented by expression of *prkC* in trans (Fig. 1*C*). Cell viability was similar at different stages of the growth curve for both the wild type and the mutant, as determined by measuring cfu (data not shown).

While the Δ*prkC2* mutant did not exhibit a general growth defect, the phylogenetic distribution of PASTA domains suggested an association of PASTA-containing proteins with peptidoglycan metabolism. For example, although our BLAST searches revealed that PrkC homologs were present in the completely sequenced genomes of nearly all low-GC-content Gram-positive bacteria, no PrkC homologs could be identified in the genomes of *Mycoplasma* spp., which cluster with the low-GC Gram-positives but lack peptidoglycan. Furthermore, most proteins that contain PASTA domains (other than PrkC homologs) are directly involved in peptidoglycan biogenesis (19) (i.e., PBPs). PASTA domains have been postulated to interact with uncross-linked peptidyl side chains of peptidoglycan, suggesting that PrkC may monitor the integrity of the *E. faecalis* cell wall, perhaps by detecting the accumulation of peptidoglycan precursors or uncross-linked peptidoglycan polymers. In response to such perturbations, we hypothesize that PrkC modulates cellular functions to ameliorate wall damage and maintain envelope integrity. Therefore, we proposed that loss of PrkC function would result in enhanced sensitivity to antibiotics that perturb cell-wall biosynthesis because of the inability to mobilize appropriate cellular adaptive responses. To test this hypothesis, we determined minimal

Table 1. Antibiotic sensitivity of the *E. faecalis* Δ*prkC2* mutant, relative to wild type, cultured in MM9YE medium supplemented with 0.2% ribose

Antibiotic	Median fold sensitization
Cell wall	
Cefuroxime	128
Ceftazidime	128
Cefotaxime	768
Ceftriaxone	128
Bacitracin	2
Vancomycin	2
Ampicillin	2
Ribosome	
Kanamycin	0.75
Gentamicin	0.75
Chloramphenicol	1
Tetracycline	1
Erythromycin	0.75

Paired MIC determinations for the wild type (OG1RF) and Δ*prkC2* mutant (CK119) were made in four independent experiments; the median fold sensitization is reported, calculated as MIC (wild type)/MIC (mutant). Values from the individual experiments are available in SI.

inhibitory concentrations (MICs) for the Δ*prkC2* mutant and the congenic wild-type strain against a collection of antimicrobial agents with various mechanisms of action and molecular targets. Essentially no difference in MIC between the strains was observed for antibiotics that target ribosomal function, including kanamycin, gentamicin, chloramphenicol, erythromycin (Em), and tetracycline (Table 1), indicating that ribosomal function is not impaired in the mutant. In contrast, MICs for antibiotics targeting distinct steps of cell-wall biosynthesis were reproducibly higher for wild-type *E. faecalis* than for the Δ*prkC2* mutant (Table 1). In particular, the most substantial effects were observed with broad-spectrum cephalosporins, including cefuroxime, ceftazidime, cefotaxime, and ceftriaxone (the mutant was >100-fold more sensitive than wild type), antibiotics to which *E. faecalis* exhibits intrinsic high-level resistance. Cephalosporin sensitivity could be almost entirely complemented by expression of *prkC* in trans (Table 2), indicating that the Δ*prkC2* mutation was responsible for the defect. For unknown reasons, cephalosporin resistance was not quite fully restored to wild-type levels in the complementation experiments. We suspect that this is related to suboptimal levels of *prkC* expression, but we currently lack a PrkC-specific antibody with which to assess PrkC levels in the cell. In addition to broad-spectrum cephalosporin sensitivity, the Δ*prkC2* mutant also reproducibly exhibited enhanced sensitivity to other cell-envelope-active antibiotics, such as bacitracin, vancomycin, and ampicillin (Table 1), although the magnitudes of the effects were more modest (2-fold sensitization). Similar results were obtained when cells were grown in BHI [see supporting information (SI)], indicating that the composition of the growth medium was not related to the sensitivity of the mutant to cell-wall-active antibiotics.

Table 2. Complementation of cephalosporin sensitivity of the Δ*prkC2* mutant in MM9YE supplemented with ribose (0.2%), Em, and rhamnose (1%)

Antibiotic	Median fold sensitization*	
	Vector control†	*prkC*⁺ complementation‡
Cefuroxime	192	6
Ceftazidime	128	4
Cefotaxime	768	5
Ceftriaxone	128	2

*Paired MIC determinations for the indicated strains were made in five or six independent experiments; the median fold sensitization (calculated as indicated) is reported. Values from the individual experiments are available in SI.

†Calculated as MIC (wild type + vector)/MIC (Δ*prkC2* + vector). Vector was pCJK96.

‡Calculated as MIC (wild type + vector)/MIC (Δ*prkC2* + *prkC*). Vector was pCJK96, and *prkC* expression plasmid was pCJK104.

The observation that not all β-lactam antibiotics (such as ampicillin) exert an effect of similar magnitude as the cephalosporins (which inhibit the same chemical reaction, transpeptidation) may initially seem counterintuitive. However, we note that ampicillin inhibits transpeptidation in *E. faecalis* with different enzymatic specificities than do the broad-spectrum cephalosporins: ampicillin inhibits all PBPs of *E. faecalis* effectively (resulting in relatively low MICs of 2–8 μg/ml), whereas broad-spectrum cephalosporins exhibit low affinity for the intrinsic enterococcal PBP5 (29, 30). PBP5 can therefore carry out transpeptidation in the presence of these agents, resulting in high-level enterococcal resistance (MICs >128 μg/ml). Thus, we speculate that the particular physiological conditions experienced by *E. faecalis* upon exposure to broad-spectrum cephalosporins, in which PBP5 is functional but the other PBPs are inhibited, effectively lead to the production of a signal recognized by PrkC. Because other β-lactams inhibit all *E. faecalis* PBPs, these antibiotics may not be as effective at producing such a signal as the broad-spectrum cephalosporins. Whatever the explanation, collectively these results suggest that PrkC is required for *E. faecalis* to detect perturbations in the cell wall or its biosynthesis and mediate adaptive responses that promote cell-wall homeostasis and enhance intrinsic resistance to cell-wall-active antibiotics.

Given the cell-envelope-related phenotypic defects of the Δ*prkC2* mutant, we asked whether mutant cells exhibited any gross morphological defects consistent with defects in cell-wall integrity. Scanning electron microscopy was used to examine cells of the Δ*prkC2* mutant and its congenic wild-type parent grown on the surface of transwell membranes. For these analyses, bacteria were cultured in two different growth media: BHI, in which growth of the two strains was identical (Fig. 1*B*); and tryptic soy broth, in which the Δ*prkC2* mutant exhibits a substantial growth defect (Fig. 1*C*) relative to the wild type. When cultured in BHI, no obvious differences in cellular morphology between the mutant and wild type were apparent (Fig. 2 *A* and *B*), consistent with the identical growth kinetics. When cultured in tryptic soy broth, nearly all wild-type cells were intact and exhibited ovoid or diplococcal morphology typical of enterococci (Fig. 2*C*). In contrast, many cells of the Δ*prkC2* mutant (≈10%) exhibited obvious and significant morphological defects when cultured in tryptic soy broth, including cell ghosts with massive lesions in the cell wall (Fig. 2*D*, arrows) and cells that appeared to have collapsed (Fig. 2*D*, arrowhead), indicating that the integrity of the mutant cell wall is compromised relative to the wild type (additional fields can be found in SI). Thus, although the nature of the stress experienced by *E. faecalis* cells growing in tryptic soy broth is unknown, PrkC appears to be required to ameliorate this stress and maintain cell-envelope integrity.

Given the impaired ability of the Δ*prkC2* mutant to respond to cell-wall stress, we investigated the effects of other ecologically relevant antimicrobial agents (i.e., bile) that perturb the cell envelope. Enterococci are often differentiated from other (non-enteric) streptococci based on their ability to grow on bile-esculin agar. Phenotypic analysis of the Δ*prkC2* mutant revealed that its growth was inhibited on bile-esculin agar (data not

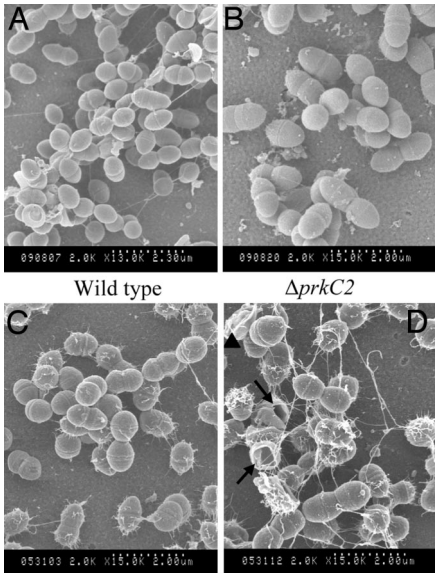

Fig. 2. Analysis of cell morphology by scanning electron microscopy. Representative fields are shown. Wild-type *E. faecalis* OG1RF (*A* and *C*) or the Δ*prkC2* mutant CK119 (*B* and *D*) were cultivated in BHI (*A* and *B*) or tryptic soy broth (*C* and *D*) on transwell membranes. Numerous collapsed cells (arrowhead) and cells with lesions in the cell wall (arrows) were apparent when the Δ*prkC2* mutant was cultivated in tryptic soy broth. Additional fields are available in SI.

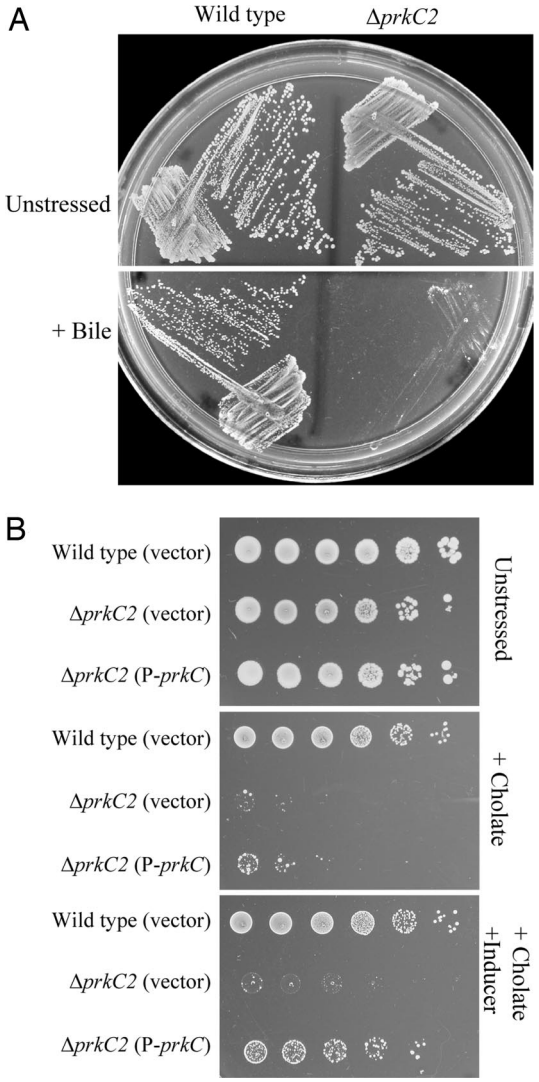

Fig. 3. The Δ*prkC2* mutant is inhibited by bile detergents. (*A*) Analysis of growth in the presence of bile. Wild-type *E. faecalis* OG1RF (left) or the Δ*prkC2* mutant CK119 (right) were cultivated on BHI agar (upper) or BHI agar supplemented with 4% unfractionated bile (lower). (*B*) Analysis of growth and complementation of the Δ*prkC2* mutant. Serial 10-fold dilutions were prepared from stationary-phase cultures carrying either an empty plasmid vector (pCJK96) or the complementation plasmid bearing *prkC* (pCJK104). Aliquots of successive dilutions were spotted from left to right on BHI agar supplemented with 10 μg/ml Em and either sodium cholate (2%) or sodium cholate plus the inducer for *prkC* expression (rhamnose, 2%), as indicated.

shown) as well as BHI agar (Fig. 3*A*) or MM9YEG agar (data not shown) supplemented with unfractionated bile. Bile is a complex mixture whose principal antimicrobial components are bile salt detergents, including cholic acid, deoxycholic acid, and their conjugated derivatives (31). Further analysis revealed that sensitivity of the Δ*prkC2* mutant and its congenic wild type to sodium deoxycholate was similar (MICs: wild type, 0.1%; Δ*prkC2* mutant, 0.075%), but that the mutant was substantially more sensitive to sodium cholate (MICs: wild type, 6%; Δ*prkC2* mutant, 1%) and sodium glycocholate, paralleling the sensitivity to unfractionated bile. The phenotypic defect of the mutant could be complemented by inducible expression of *prkC* in trans (Fig. 3*B*), confirming that the Δ*prkC2* mutation was responsible for the defect. Using disk-diffusion assays, we also found that the Δ*prkC2* mutant exhibited increased sensitivity to the detergent SDS (data not shown). Thus, PrkC appears to be a general sensor of cell-envelope stress in *E. faecalis*.

To determine whether PrkC-mediated resistance to bile conferred a persistence advantage in the intestinal tract, we orally inoculated mice with equal viable cfu of either the Δ*prkC2* mutant or its congenic wild-type parent. The following day, intestinal persistence of these strains was assessed by plating cecal contents on selective agar media. In these experiments, high-level intestinal colonization with orally inoculated strains cannot be achieved because of strong colonization resistance from the indigenous flora. Despite this competition, substantial numbers of wild-type *E. faecalis* could be recovered from the cecum after 16 h (Table 3). Persistence of the Δ*prkC2* mutant was reduced 6-fold (statistically significant difference) at the same time point, indicating that PrkC function contributes to persistence in the intestinal tract. We speculate that the loss of PrkC-mediated resistance to bile is responsible for the impaired ability to persist in the mouse intestine.

Concluding Remarks

The bipartite eSTK PrkC is a member of a new class of one-component signal transduction proteins that mediates adaptive responses to cell-envelope stress in *E. faecalis*. The genome of *E. faecalis* OG1RF (the strain from which *prkC* was deleted in this study) lacks any known mobile genetic elements, and *prkC* is also encoded in the genome of the sequenced strain of *E. faecalis*, V583. In the V583 genome, no obvious mobile genetic elements are found in the vicinity of *prkC*. Thus, PrkC is apparently encoded by the core genome of *E. faecalis* and regulates adaptive responses that confer the inherent resistance to cell-envelope stress that is characteristic of the species. Furthermore, these traits are associated with the success of *E. faecalis* as a major nosocomial pathogen. By conferring intrinsic resistance to antimicrobials and contributing to the ecological success of *E. faecalis* in the intestinal tract, PrkC facilitates high-level colonization with antibiotic-resistant enterococci that precedes the onset of infection. Selectively eliminating such enterococci from the intestine may be an effective strategy for

Table 3. Aerobic and facultative cecal bacteria (average cfu ± SE $\log_{10}$ per gram) in mice (n = 8 per group) orally inoculated with *E. faecalis* strains

Oral *E. faecalis* strain inoculated	Groups of cecal bacteria: Inoculated *E. faecalis* strain	Total *E. faecalis**	Total Gram-positive	Total Gram-negative
None	None detected	5.8 ± 0.5	5.6 ± 0.6	4.4 ± 0.5
Wild type	5.4 ± 0.1	6.1 ± 0.3	6.6 ± 0.4	5.3 ± 0.4
Δ*prkC2*	4.6 ± 0.3†	5.7 ± 0.4	6.7 ± 0.5	5.4 ± 0.3

*Includes indigenous *E. faecalis* plus orally inoculated *E. faecalis*.
†Decreased 6-fold ($P < 0.01$) compared with wild-type OG1RF.

the prevention of antibiotic-resistant enterococcal infections. Therapeutic agents that inhibit PrkC function and render *E. faecalis* susceptible to the antimicrobial effects of bile are attractive candidates for such an approach.

Homologs of PrkC in some pathogenic streptococci are required for full virulence in animal models of infection (21, 23), suggesting that the streptococcal PrkC homologs regulate the expression or activity of virulence factors in their respective hosts. These streptococcal PrkC homologs each exhibit a PrkC-like bipartite domain architecture including PASTA domains, suggesting that, like *E. faecalis* PrkC, they detect perturbations of the cell envelope as sensory input. Therefore, it appears that PrkC-like signal transduction systems have been adapted to regulate species-specific biological responses to cell-envelope stress that are appropriate for a given organism in its unique ecological niche.

Materials and Methods

Bacterial Strains, Growth Media, and Chemicals. Standard *Escherichia coli* strains DH5α or XL1Blue were used for routine cloning and plasmid propagation. *E. faecalis* OG1RF (32) was used as the wild-type strain. *E. faecalis* CK119 is a congenic derivative of OG1RF bearing an in-frame deletion of *prkC*, constructed as described below. Bacto BHI and Bacto tryptic soy broth were prepared as described by the manufacturer (Becton Dickinson, Sparks, MD). MM9YEG medium, a semidefined M9-based medium, was previously described (28). In indicated cases, ribose (0.2%) was substituted for glucose in MM9YEG (because growth in glucose was found to result in apparent catabolite repression from the rhamnose-inducible promoter of pCJK104). Bacteria were stored at −80°C in BHI supplemented with 30% glycerol. Antibiotics, detergents, and other chemicals were obtained from Sigma (St. Louis, MO).

Construction of the *E. faecalis* Δ*prkC2* Mutant CK119. All PCR amplifications used *E. faecalis* OG1RF chromosomal DNA as a template, with primers designed on the basis of the *E. faecalis* V583 genome sequence available at The Institute for Genomic Research. The markerless exchange system described by Kristich *et al.* (28) was used to construct an unmarked, in-frame deletion of *prkC* in strain OG1RF. Briefly, a derivative of plasmid pCJK47 carrying an in-frame deletion allele of *prkC* was constructed according to the BsaI cloning scheme, as described (28), that seamlessly fused two PCR amplicons flanking *prkC* to form the in-frame deletion. The deletion allele was designed such that the first 13 codons and the last 6 codons of the *prkC* gene remained, which were retained in the construct in an effort to avoid any unanticipated effects on expression of adjacent genes. More than 97% of the *prkC* gene was eliminated in the mutant construct. This Δ*prkC2* allele was transferred to the native *prkC* location in the *E. faecalis* OG1RF chromosome as previously described (28).

Construction of a Plasmid for Complementation of the Δ*prkC2* Mutation. A plasmid for rhamnose-inducible expression of *prkC* in trans (pCJK104) was constructed as follows. Plasmid pMSP3535 (33) was digested with BglI/BsaBI to excise the *nisRK* genes. T4 polymerase was used to make the ends blunt, and the vector backbone was self-ligated to produce plasmid pCJK95. A PCR amplicon encompassing a putative rhamnose-inducible promoter and adjacent transcriptional regulator (identified by searching the *E. faecalis* V583 genome sequence) was obtained with primers designed according to the *E. faecalis* V583 genome sequence and *Pfu* ultra polymerase (Stratagene, La Jolla, CA) by using *E. faecalis* OG1RF chromosomal DNA as a template. This amplicon was cloned into pCJK95 by using the primer-encoded BglII/SpeI restriction sites, creating plasmid pCJK96. Preliminary experiments in which *lacZ* was cloned under the control of the putative rhamnose-inducible promoter encoded in pCJK96 demonstrated rhamnose-inducible production of β-galactosidase (data not shown). Therefore, the *prkC* ORF was amplified from OG1RF chromosomal DNA with *Pfu* ultra polymerase and cloned into pCJK96 by using primer-encoded SphI/XbaI restriction sites, creating pCJK104 in which *prkC* was under the control of the rhamnose-inducible promoter.

Analysis of Unstressed Growth. Stationary-phase overnight cultures in BHI or tryptic soy broth supplemented with 10 μg/ml Em (for plasmid maintenance) and 2% rhamnose (for induction of *prkC*) were diluted to an optical density of 0.002 at 600 nm in fresh, prewarmed corresponding growth medium. Cultures were incubated aerobically at 37°C under static conditions with occasional mixing. Growth was monitored by removing aliquots for optical density measurements (600 nm) at intervals.

Tests of Antibiotic Sensitivity. MICs for antibiotics were determined in aerobic liquid cultures by using a microtiter plate serial dilution method. Two-fold dilutions of antibiotics in MM9YE (supplemented with ribose as a carbon source) or BHI were prepared in the wells of a 96-well microtiter plate. Bacteria from stationary-phase cultures in corresponding growth medium were inoculated into each well to a concentration of $\approx 10^5$ cfu/ml. Microtiter plates were incubated at 37°C on an orbital shaker (New Brunswick Scientific, Edison, NJ) Model G2 at 225 rpm for 24 h. The lowest concentration of antibiotic that prevented growth was recorded as the MIC. For antibiotic sensitivity complementation experiments, 10 μg/ml Em (for plasmid maintenance) and 1% rhamnose (for induction of *prkC*) were included in the microtiter plate growth medium. MICs for sodium cholate and sodium deoxycholate were determined on BHI agar plates by spotting $\approx 2 \times 10^4$ cfu from stationary-phase cultures in BHI and incubating at 37°C for 24 h. The lowest concentration of detergent that prevented growth was recorded as the MIC. Sodium cholate was tested in 0.5% increments over a range from 0% to 7%. Sodium deoxycholate was tested in 0.025% increments over a range from 0% to 0.1%.

Scanning Electron Microscopy. Previous studies found that growth of *E. faecalis* on uncoated membranes of transwell culture environments (Becton Dickinson) is a convenient experimental system for SEM analysis of enterococcal cell morphology and extracellular polymer production (G.M.D., unpublished data). Therefore, this system was used to examine the morphology of the Δ*prkC2* mutant. Strains of *E. faecalis* were cultivated in BHI or tryptic soy broth in transwells at 37°C for 3 days, with daily replacement of the spent medium with fresh medium as previously described (34). Sample fixation and processing were as previously described (34). Briefly, samples were fixed in aldehydes supplemented with 0.15% alcian blue. After primary fixation, the samples were washed in 0.15 M cacodylate buffer and postfixed for 90–120 min in 1% OsO_4 in 0.15 M cacodylate buffer containing 1.5% potassium ferrocyanide. Samples were then rinsed in cacodylate buffer and dehydrated in an ascending ethanol series [50%, 70%, 80%, 95%, and 100% (twice)] before critical point drying with CO_2. Specimens were mounted on adhesive carbon films and then coated with ≈1 nm of platinum by using an Ion Tech argon ion beam coater. Examination of samples was performed with a Hitachi S-900 SEM.

Intestinal Persistence in Mice. Female Swiss–Webster mice (18–22 g; Harlan Sprague–Dawley, Indianapolis, IN) were allowed to acclimatize 1 week before experiments. Experiments were performed according to the National Institutes of Health guidelines, and the University of Minnesota Institutional Animal Care and Use Committee approved the protocol. Untreated mice were orally inoculated (feeding needle) with 0.1 ml containing saline or 5×10^8 washed cells (overnight tryptic soy broth culture) of *E. faecalis* OG1RF or *E. faecalis* CK119 suspended in 0.1 ml of sterile saline. Viable cfu in the inocula were determined by serial dilution and plating for enumeration to verify that wild-type and mutant inocula contained equal viable cell numbers. Separate groups of four mice were given the same inoculum 24 h and 16 h before sacrifice by cervical dislocation. Ceca were aseptically excised, serially diluted, and plated on MacConkey agar (for selective isolation of Gram-negative bacilli), colistin nalidixic acid agar supplemented with 5% sheep red blood cells (for selective isolation of Gram-positive bacteria), and BHI agar supplemented with 200 μg/ml rifampin (for selective isolation of *E. faecalis* OG1RF and CK119 away from indigenous rifampin-sensitive *E. faecalis*). Experiments were replicated on separate days, and data were pooled. Statistical analyses were performed with StatView 5.0.1 (SAS Institute, Cary, NC). Numbers of cecal microbes were converted to $\log_{10}$ and analyzed by analysis of variance followed by Fisher's post hoc test. Statistical significance was $P \leq 0.05$.

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