Reciprocal Regulation of Cephalosporin Resistance in Enterococcus faecalis

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ABSTRACT Antibiotic-resistant enterococci are major causes of hospital-acquired infections and therefore represent a serious public health problem. One well-known risk factor for the acquisition of hospital-acquired enterococcal infections is prior therapy with broad-spectrum cephalosporin antibiotics. Enterococci can proliferate in patients undergoing cephalosporin therapy due to intrinsic cephalosporin resistance, a characteristic of the genus Enterococcus. However, the molecular basis for cephalosporin resistance in E. faecalis has yet to be adequately elucidated. Previously we determined that a putative Ser/Thr kinase, IreK (formerly PrkC), is required for intrinsic cephalosporin resistance in E. faecalis. Here we show that kinase activity is required for cephalosporin resistance and, further, that resistance in E. faecalis is reciprocally regulated by IreK and IreP, a PP2C-type protein phosphatase encoded immediately upstream of IreK. Mutants of two divergent lineages of E. faecalis lacking IreP exhibit remarkable hyperresistance to cephalosporins but not to antibiotics targeting other cellular processes. Further genetic analyses indicate that hyperresistance of the IreP mutant is mediated by the IreK kinase. Additionally, competition experiments reveal that hyperresistant ΔireP mutants exhibit a substantial fitness defect in the absence of antibiotics, providing an evolutionary rationale for the use of a complex signaling system to control intrinsic cephalosporin resistance. These results support a model in which IreK and IreP act antagonistically via protein phosphorylation and dephosphorylation as part of a signal transduction circuit to regulate cellular adaptation to cephalosporin-induced stress.

IMPORTANCE As a major cause of hospital-acquired infections, antibiotic-resistant enterococci represent a serious public health problem. Enterococci are well-known to exhibit intrinsic resistance to broad-spectrum cephalosporin antibiotics, a trait that enables them to proliferate in patients undergoing cephalosporin therapy, thereby predisposing these patients to acquisition of an enterococcal infection. Thus, inhibition of enterococcal cephalosporin resistance could represent an effective new strategy to prevent the emergence of hospital-acquired enterococcal infections. At this time, however, the molecular basis for cephalosporin resistance in E. faecalis is poorly understood. Our results begin to unravel the details of a new phosphorylation-dependent signal transduction system that controls cephalosporin resistance in enterococci. Deeper understanding of the mechanism underlying cephalosporin resistance in E. faecalis may enable the development of new therapeutics designed to reduce the incidence of hospital-acquired enterococcal infections.

The Gram-positive bacterium Enterococcus faecalis is a commensal inhabitant of the gastrointestinal tracts of a wide variety of insects and animals, including humans (1). However, antibiotic-resistant enterococci are also major causes of hospital-acquired infections (2) and therefore represent a serious public health problem. One well-known risk factor for the acquisition of enterococcal hospital-acquired infections is prior therapy with broad-spectrum cephalosporins (3), antibiotics that belong to the β-lactam family and interfere with cell wall biosynthesis by inhibiting the penicillin-binding proteins (PBPs) that cross-link peptidoglycan (PG). The prevailing model to explain the association of cephalosporin resistance with increased risk of enterococcal infection (reviewed in reference 3) invokes the observation that enterococci proliferate to achieve abnormally high densities in the gastrointestinal (GI) tract of patients during cephalosporin therapy (4), a situation that likely facilitates enterococcal dissemination to other sites and the subsequent emergence of infection. Proliferation of enterococci during cephalosporin therapy is possible because enterococci exhibit intrinsic resistance to broad-spectrum cephalosporins. This intrinsic cephalosporin resistance is a trait shared by essentially all isolates of E. faecalis, yet our understanding of the genetic and biochemical basis underlying cephalosporin resistance remains incomplete.

Three genetic loci have thus far been reported to be critical for cephalosporin resistance in E. faecalis: (i) pnp5, which encodes a so-called “low-affinity” PBP that exhibits reduced affinity for cephalosporins and presumably retains the ability to synthesize cell wall despite the presence of cephalosporins in the environment (5, 6); (ii) a locus encoding a two-component signal transduction system (CroRS) which presumably regulates the expression of as-yet-unknown genes that promote cephalosporin resistance (7, 8); and (iii) a gene encoding a predicted Ser/Thr...
kinase, genetic deletion of which drastically reduces cephalosporin resistance but which has not yet formally been shown to possess kinase activity (9). In our original report (9), this kinase was referred to as PrkC—here we rename it IreK (for intrinsic resistance of enterococci), to reflect its critical role in intrinsic cephalosporin resistance.

IreK belongs to a family of Ser/Thr protein kinases with a characteristic bipartite domain architecture. Kinases in this family share a presumably cytoplasmic kinase domain separated by a predicted transmembrane segment from a series of PASTA domains that are thought to bind PG or fragments thereof (10–12). Homologs of IreK with similar domain architecture are present in the genomes of nearly all low-GC Gram-positive bacteria (usually found in 1 copy per genome), and analyses of mutants lacking those kinases have revealed diverse functional roles for the kinases, including development of competence, regulation of intra- and extracellular nucleotide pools, virulence, control of hemolysin production, cell division, stationary-phase survival, germination of endospores, and modulation of antibiotic resistance (reviewed in reference 13).

Although the phenotypic consequences of genetic lesions in the kinases have been studied in some detail for numerous Gram-positive bacteria, less is known about mechanisms of kinase regulation. Extensive structural studies on the purified kinase domain of the mycobacterial IreK homolog (PknB) revealed a back-to-back homodimer (14, 15) and suggested a regulatory mechanism by which dimerization allosterically activates the kinase (16). Additionally, mass spectrometry studies have revealed that the purified kinase domains of two IreK homologs (mycobacterial PknB and Bacillus subtilis PrkC) can be autophosphorylated at multiple sites per monomer—in most cases, at threonine residues—including at several conserved sites in the kinase “activation loop” (14, 17–19). The activation loop is a short, centrally located segment of the kinase domain that is thought to undergo a conformational shift upon phosphorylation, leading to activation of the kinase. Substitution of the phosphorylatable residues with alanine in the kinase domains of PknB and PrkC substantially reduces kinase activity (17, 18), suggesting that autophosphorylation of the activation loop indeed enhances kinase activity for both PknB and PrkC. Thus far, the vast majority of studies probing the mechanisms of kinase regulation have been performed in vitro using purified kinase domains. Although it seems likely that these findings will translate into the in vivo setting, at present it is not clear to what extent these mechanisms contribute to kinase regulation in vivo.

In the genomes of most Gram-positive bacteria, encoded immediately upstream of the IreK homolog is a protein Ser/Thr kinase, and homologs of IreK from other Gram-positive bacteria have been experimentally shown to be kinases (13). To test if E. faecalis IreK does indeed exhibit kinase activity, we purified a recombinant 6His-tagged fragment of IreK corresponding to the entire N-terminal fragment containing the Ser/Thr kinase domain and juxtamembrane region (IreK-n) and performed in vitro kinase assays using myelin basic protein (MBP) as a surrogate substrate (MBP is routinely used as a substrate for IreK homologs from other species of Gram-positive bacteria). We also analyzed a mutant of IreK-n bearing a lysine-to-arginine substitution (K41R in IreK) at a conserved lysine within the kinase ATP-binding P loop. Mutations at this invariant lysine residue in other kinases of the IreK family are known to significantly impair kinase activity (19, 22, 28, 29). Using the phosphoprotein stain ProQ Diamond, we found that wild-type IreK-n phosphorylates MBP in the presence of ATP. Furthermore, IreK-n itself exhibited a strong signal, suggesting that it was autophosphorylated as well (Fig. 1A). The IreK-n K41R mutant was substantially impaired at phosphorylation of MBP and itself exhibited a significantly reduced phosphoprotein signal. In addition, we observed that the K41R mutant exhibited a subtle shift in electrophoretic mobility to a species that migrated faster through the gel than wild-type IreK-n, suggesting that IreK autophosphorylation resulted in reduced electrophoretic mobility. We conclude that, as expected, E. faecalis IreK is indeed a protein kinase.

Because the E. faecalis ΔIreK mutant is markedly susceptible to cephalosporins, the IreK protein must play a role in promoting cephalosporin resistance. To test if this phenotype reflects a requirement for the kinase activity of IreK or for the IreK protein per se, we expressed either the full-length wild-type or K41R mu-

### RESULTS

**E. faecalis IreK is a protein kinase whose activity is required for cephalosporin resistance.** Genetic analysis described in a previous study (9) revealed that IreK is required for intrinsic cephalosporin resistance in E. faecalis. Sequence analysis predicted IreK to be a Ser/Thr protein kinase, and homologs of IreK from other Gram-positive bacteria have been experimentally shown to be kinases (13). To test if E. faecalis IreK does indeed exhibit kinase activity, we purified a recombinant 6His-tagged fragment of IreK corresponding to the entire N-terminal fragment containing the Ser/Thr kinase domain and juxtamembrane region (IreK-n) and performed in vitro kinase assays using myelin basic protein (MBP) as a surrogate substrate (MBP is routinely used as a substrate for IreK homologs from other species of Gram-positive bacteria). We also analyzed a mutant of IreK-n bearing a lysine-to-arginine substitution (K41R in IreK) at a conserved lysine within the kinase ATP-binding P loop. Mutations at this invariant lysine residue in other kinases of the IreK family are known to significantly impair kinase activity (19, 22, 28, 29). Using the phosphoprotein stain ProQ Diamond, we found that wild-type IreK-n phosphorylates MBP in the presence of ATP. Furthermore, IreK-n itself exhibited a strong signal, suggesting that it was autophosphorylated as well (Fig. 1A). The IreK-n K41R mutant was substantially impaired at phosphorylation of MBP and itself exhibited a significantly reduced phosphoprotein signal. In addition, we observed that the K41R mutant exhibited a subtle shift in electrophoretic mobility to a species that migrated faster through the gel than wild-type IreK-n, suggesting that IreK autophosphorylation resulted in reduced electrophoretic mobility. We conclude that, as expected, E. faecalis IreK is indeed a protein kinase.

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IreK kinase activity is required for cephalosporin resistance in *E. faecalis*. (A) *In vitro* kinase activity of IreK. Wild-type (WT) and mutant (K41R) IreK kinase domains were purified and used for *in vitro* phosphorylation reactions with myelin basic protein (MBP) as a surrogate substrate. Reaction mixtures were incubated in the absence (−) or presence (+) of 2 mM ATP. At the indicated times (in minutes), aliquots were quenched with SDS loading buffer and subjected to SDS-PAGE. Phosphoproteins were detected using ProQ Diamond phosphoprotein stain, followed by GelCode blue staining to detect total proteins. Molecular weight standards are indicated at the left. Results are representative of a minimum of three independent experiments. (B) Kinase activity is required for resistance. Cultures of plasmid-bearing strains were subjected to serial 10-fold dilutions and inoculated (left to right, least to most dilute) onto BHI agar supplemented with Em alone (control) or in addition to cephalexin, 1 μg/ml. The WT and ΔirePK strains were OG1RF and CK125, respectively. Plasmids are indicated in parentheses: vector, pJRG8 empty vector; WT, pCJK160 expressing wild-type IreP and IreK; K41R, pCJK216 (analogous to pCJK160 but carrying the K41R allele of ireK). (C) Immunoblot analysis of IreK expression. Whole-cell lysates from CK125 (ΔirePK) carrying empty vector (pJR88) or pJR88 expressing IreP and either wild-type ireK (pCJ160) or ireK K41R (pCJ216) were probed with anti-IreK antibody (α-kinase) or anti-sigma factor antibody (α-sigma) as a loading control. Results are representative of a minimum of two experiments analyzing at least three transformants derived from independent electroporations.

** FIG 1 **

IreP is a phosphatase that can dephosphorylate IreK. Recombinant His-tagged IreP was purified and used for *in vitro* phosphatase reactions. (A) Phosphatase activity monitored using *p*-nitrophenyl phosphate (pNPP) as a substrate. IreP was incubated with pNPP and various concentrations of either Mg\(^{2+}\) or Mn\(^{2+}\) as a cofactor for 20 min at room temperature. The reactions were quenched, and absorbance was measured at 405 nm to detect cleaved product. Error bars represent standard deviations for triplicate samples and are too small to see in some cases. (B) IreP was incubated in kinase buffer with ATP and MBP for 30 min to allow phosphorylation to occur. The reaction mixture was split, and IreP was added to 1 aliquot; mixtures were incubated for 30 min and subjected to SDS-PAGE. Phosphoproteins were detected using ProQ Diamond phosphoprotein stain followed by GelCode blue staining to detect total proteins. Results are representative of a minimum of three independent experiments.

** FIG 2 **

E. faecalis IreP is a protein phosphatase that can dephosphorylate the IreK kinase. In most low-GC Gram-positive bacteria, the gene located immediately upstream of the IreK kinase homolog is cotranscribed and encodes a protein phosphatase of the PP2C family. The genetic organization of the *ireK* locus in *E. faecalis* is similar, with a putative protein phosphatase (here named IreP) encoded upstream of, and slightly overlapping, the gene for the *ireK* kinase. We tested for cotranscription of *ireP* and *ireK* by isolating RNA from wild-type *E. faecalis* and performing reverse transcription-PCR analysis. We observed evidence for a transcript containing both *ireP* and *ireK* (data not shown), indicating that the kinase and phosphatase are cotranscribed in *E. faecalis*. Our data also indicate that the *ireP*-ireK pair can be cotranscribed with the genes immediately upstream of *ireP* (a putative rRNA methyltransferase; EF3122) and downstream of *ireK* (a putative GTPase; EF3119). Of note, the *B. subtilis* homolog of IreK (PrkC) has been reported to phosphorylate the GTPase encoded upstream of, and slightly overlapping, the gene for the *ireK* kinase. However, our data do not indicate if this is functionally significant (30). We attempted to phosphorylate the *E. faecalis* homolog (EF3119) with IreK-n *in vitro* but did not observe any evidence for phosphorylation under our conditions (data not shown).

To test if *E. faecalis* IreP exhibited phosphatase activity, we purified a recombinant 6His-tagged IreP and performed *in vitro* phosphatase assays using the small-molecule colorimetric phosphatase substrate *p*-nitrophenyl phosphate, which forms a colored product upon dephosphorylation. *E. faecalis* IreP exhibited phosphatase activity in a manganese-dependent manner (Fig. 2A), consistent with the properties of IreP homologs from other Gram-positive bacteria. We attempted to phosphorylate the *E. faecalis* homolog (EF3119) with IreK-n *in vitro* but did not observe any evidence for phosphorylation under our conditions (data not shown).
other Gram-positive bacteria. *E. faecalis* IreP was also capable of dephosphorylating the IreK substrate MBP and, importantly, IreK- n kinase itself (Fig. 2B). In addition, IreP treatment resulted in a subtle shift in electrophoretic mobility of IreK-n that appears to be characteristic of a change in phosphorylation status. We note that IreP itself exhibited a faint phosphoprotein signal; however, control experiments in the absence of ATP (and on IreP purified directly from *E. coli*) indicated that this signal represents nonspecific background staining of IreP (not shown).

**E. faecalis ΔireP mutants exhibit hyperresistance to cephalosporins.** We hypothesized that IreP-mediated dephosphorylation of IreK in vivo might play a critical regulatory role in controlling IreK kinase activity and, by extension, cephapmorin resistance. To probe the role of IreP in vivo, we constructed *E. faecalis* mutants lacking the *ireP* gene. In this context, it is noteworthy that IreP is the only identifiable PP2C-family phosphatase encoded in the *E. faecalis* genome. Because *ireP* overlaps with *ireK*, we constructed an in-frame deletion lacking 92% of the *ireP* gene without disrupting any *ireK* coding sequences, to avoid perturbing expression of the *ireK* kinase. Immunoblot analysis verified that IreK was indeed expressed in the ΔireP mutant at levels comparable to that of wild-type (Fig. 3B). On brain heart infusion (BHI) agar plates, colonies of the ΔireP mutants exhibited a distinct morphology, appearing more opaque (white) and compact than those of the isogenic wild type. Exponential-growth rates for the ΔireP mutant in liquid culture were only slightly lower than those for the wild type (generation times of 31 ± 2 min versus 37 ± 2 min in Mueller-Hinton broth [MHB] for the wild type [CK138] and the isogenic ΔireP mutant [CK204], respectively). However, antimicrobial susceptibility tests revealed a striking phenotype: the ΔireP mutant was substantially more resistant to cephapmorins (>64-fold for ceftriaxone) than the isogenic wild-type strain (Fig. 3A; Table 1). Indeed, the ΔireP mutant was capable of growth at all cephapmorin concentrations tested (up to 2,048 μg/ml). Expression of *ireP* in trans eliminated hyperresistance (Fig. 3C), indicating that hyperresistance was indeed due to the lesion in *ireP*. Furthermore, we constructed an identical deletion of *ireP* in a divergent lineage of *E. faecalis* (T1) and found that the *E. faecalis* T1 ΔireP mutant also exhibited a cephapmorin hyperresistance phenotype relative to its isogenic wild-type parent (Fig. 3A). Thus, the role of IreP in regulating cephapmorin resistance is likely conserved across *E. faecalis* as a species.

Previous work established that *E. faecalis* requires the IreK kinase for its intrinsic resistance to cephapmorins but not for resistance to antibiotics affecting other cellular processes. Because we hypothesized that IreP functions to regulate the activity of the IreK

**FIG 3** *E. faecalis* ΔireP mutants exhibit hyperresistance to cephapmorins. (A) Cultures were subjected to 10-fold serial dilutions and inoculated (left to right, least to most dilute) on BHI agar supplemented with indicated concentrations of ceftriaxone. Strains: OG, wild-type *E. faecalis* OG1RF; OG ΔireK, CK119; OG ΔireP, CK121; T1, wild-type *E. faecalis* T1; T1 ΔireK, JL202; T1 ΔireP, JL204. (B) Immunoblot analysis of IreK expression. Whole-cell lysates from OG1RF (wild-type), CK119 (ΔireK), CK125 (ΔireP ΔireK), and CK121 (ΔireP) were probed with anti-IreK antibody (α-kinase) or anti-sigma factor antibody (α-sigma) as a loading control. (C) Complementation analysis of the *E. faecalis* ΔireP mutant. Cultures of plasmid-bearing strains were subjected to serial 10-fold dilutions and inoculated (left to right, least to most dilute) onto BHI agar supplemented with Em alone (control) or in addition to ceftriaxone. WT and ΔireP strains were OG1RF and CK121. Plasmids are indicated in parentheses: vector, pJRG8 empty vector; *ireP*, pJL225 expressing wild-type IreP. Results are representative of a minimum of three experiments analyzing independently derived mutants.

TABLE 1 Median MICs for wild-type and mutant *E. faecalis* strains

| Drug type or target and name | MIC (μg/ml)× for: |
|-----------------------------|--------------------|
|                             | OG1RF (wild type) | CK119 (ΔireK) | CK121 (ΔireP) | CK123 (ΔireP ΔireK) |
| Cephalosporins               |                    |                |                |                    |
| Ceftriaxone                  | 32                 | 2              | >2,048         | 2                  |
| Cefazidime                   | 128                | 16             | >2,048         | 16                 |
| Other cell wall              |                    |                |                |                    |
| Aminoglycoside               | 1                  | 2              | 2              | 0.5                |
| Vancomycin                   | 2                  | 1              | 1              | 1                  |
| Bacitracin                   | 64                 | 32             | 32             | 32                 |
| D-Cycloserine                | 128                | 64             | 256            | 64                 |
| Other targets                |                    |                |                |                    |
| Norflaxacin                  | 4                  | 4              | 2              | 4                  |
| Chloramphenicol              | 4                  | 4              | 4              | 4                  |
| Kanamycin                    | 128                | 128            | 64             | 128                |

× Determined in MHB after 24 h incubation at 37°C from a minimum of three independent experiments.
kinase, we reasoned that the ΔireP mutant would likewise exhibit hyperresistance specifically towards cephalosporins. To test this, we performed susceptibility analyses with antibiotics targeting various cellular functions. The results (Table 1) indicate that the hyperresistant phenotype of the ΔireP mutant is essentially specific for cephalosporins, as few changes in susceptibility to other antibiotics were apparent. Thus, hyperresistance of the ΔireP mutant is not the result of enhancement in a general stress response. Furthermore, these results are consistent with the hypothesis that an important role of IreP is to control IreK kinase activity, thereby influencing cephalosporin resistance.

Hyperresistance of the E. faecalis ΔireP mutant is mediated by IreK. Phosphorylation of amino acids in the kinase activation loop is known to enhance the catalytic activity of Ser/Thr kinases in the superfamily to which IreK belongs. In the absence of the IreP phosphatase, we reasoned that IreK might become highly phosphorylated, including at sites in the activation loop, leading to enhanced kinase activity that could drive hyperresistance to cephalosporins. To test this hypothesis, we observed that hyperresistance of the ΔireP mutant is a consequence of uncontrolled, abnormally high IreK kinase activity, we performed an episomal experiment by constructing a mutant of E. faecalis lacking the genes for both the ireP phosphatase and the ireK kinase. Antimicrobial susceptibility tests revealed that the double mutant phenocopied the kinase single mutant (Table 1)—removal of ireK eliminated hyperresistance exhibited by the ΔireP mutant. Indeed, no differences were observed between the phenotype of the ΔireP ΔireK double mutant and that of the kinase single mutant. Thus, these data are consistent with the hypothesis that IreP regulates cephalosporin resistance at least in part by modulating activity of the IreK kinase.

Given the observation that IreP is capable of dephosphorylating IreK in vitro (Fig. 2B), we hypothesized that IreP-mediated dephosphorylation of sites in the activation loop of the IreK kinase served to control IreK activity in vivo. To test this, we constructed a kinase allele carrying phosphomimetic (T-to-E) substitutions at the three predicted sites of phosphorylation in the IreK activation loop [ireK(T163E/T166E/T168E)]. The likely sites of phosphorylation in IreK were chosen based on sequence alignment and comparison with known sites of phosphorylation on the mycobacterial PknB and B. subtilis PkC kinases. Mass spectrometry analyses subsequently confirmed that these sites can be phosphorylated on IreK-n (C. L. Hall and C. J. Kristich, unpublished data). We reasoned that the T-to-E substitutions in IreK would mimic phosphorylation at those sites and lead to kinase activation, but because they are uncevable by IreP, the mutant kinase would exhibit constitutively high activity. We predicted that this would lead to enhanced cephalosporin resistance—indeed, similar to the phenotype of the ΔireP mutant—despite the presence of wild-type IreP phosphatase in the cells. We expressed the T-to-E triple mutant kinase along with wild-type IreP phosphatase in E. faecalis and confirmed by immunoblotting that the mutant kinase was expressed at levels comparable to that of the wild type (Fig. 4A). Antimicrobial susceptibility tests revealed that expression of the phosphomimetic-bearing kinase allele does indeed provide enhanced cephalosporin resistance relative to its wild-type counterpart (Table 2), consistent with the hypothesis that IreP dephosphorylates the activation loop of IreK kinase to negatively control its activity. We note that although the strain expressing the phosphomimetic ireK allele exhibits enhanced cephalosporin resistance, it is not as hyperresistant as the ΔireP mutant, suggesting that IreP-mediated dephosphorylation of heterologous kinase substrates—or other phosphorylated sites on the kinase itself—are also important for regulation of cephalosporin resistance in vivo. Alternatively, the T-to-E substitutions may not fully mimic the effect of phosphorylation at those sites.

Evidence for modulation of IreP phosphatase activity by IreK. The experiments described above suggest that an important role of IreP is to negatively control IreK kinase activity via dephosphorylation of the kinase activation loop. However, this inhibitory activity is counterproductive when E. faecalis is confronted with cephalosporins, in which case enhanced kinase activity is desirable to mediate the appropriate biological response and generate resistance. Therefore, we reasoned that a mechanism might exist to transiently overcome the negative regulatory effect of IreP—attenuate the phosphatase activity of IreP—upon kinase activation. To test this, we performed phosphatase assays on lysates of E. faecalis strains, using a phosphothreonine-containing peptide as a surrogate phosphatase substrate. We compared phosphatase activity in lysates of a strain expressing wild-type IreK kinase with that in lysates of a strain expressing the hyperactive (phosphomimetic) triple-T-to-E allele of ireK (to mimic a state of kinase activation), IreP-specific phosphatase activity in lysates from the strain containing the phosphomimetic kinase was reproducibly lower than that in lysates from a strain with a wild-type kinase (Fig. 4B), suggesting that some mechanism exists to attenuate IreP phosphatase activity, at least transiently, upon kinase activation.

Hyperresistant ΔireP mutants exhibit a fitness defect. Our data support the hypothesis that IreP and IreK comprise key elements of a signal transduction system that regulates intrinsic

![FIG 4 Reduced phosphatase activity in lysates with enhanced IreK kinase activity. (A) Immunoblot analysis of IreK expression. Whole-cell lysates from CK125 carrying an empty vector (pJR8G) or expressing IreP with either a wild-type IreK kinase (pCJK160) or the T163E/T166E/T168E triple mutant phosphomimetic allele (pCJK201) were probed with anti-IreK antibody (α-kinase) or anti-sigma factor antibody (α-sigma) as a loading control. (B) Phosphothreonine-specific phosphatase activity was measured in cleared whole-cell lysates of the strains used for panel A. Error bars represent standard errors of the means of values from three independent lysates and are too small to be seen in most cases.]
cephalosporin resistance in E. faecalis. That such a signaling system exists implies that the cellular adaptation(s) required to overcome cephalosporin stress imposes a fitness cost on the organism in the absence of cephalosporins. To test this hypothesis, we performed coculture competition experiments in which a marked wild-type E. faecalis strain (marked with an unrelated antibiotic resistance allele) was cocultured with a differentially marked mutant strain lacking the IreP phosphatase. Cocultures were inoculated with various ratios of the two strains and subjected to repeated daily cycles of growth and dilution into fresh medium lacking cephalosporins. Aliquots of the cocultures were removed at intervals and plated on appropriate selective media to distinguish wild-type E. faecalis from ΔireP mutants. The results (Fig. 5) revealed that the wild-type strains rapidly outcompeted the ΔireP mutants over the course of even a single dilution/growth cycle (day 1) and even when the inoculum (day 0) was initially composed of >90% mutants. Reciprocal competition experiments performed with strains in which the antibiotic markers were swapped (to ensure that the observed fitness defect of the ΔireP mutant was not impacted by the antibiotic markers used to tag the strains) yielded similar results (Fig. 5). Thus, mutants lacking ireP are substantially less fit in the absence of cephalosporins than wild-type E. faecalis, suggesting that IreK-mediated adaptation(s) to cephalosporin stress, while obviously beneficial in the presence of cephalosporins, is costly to the cell in environments devoid of cephalosporins.

**DISCUSSION**

The experiments described here were conducted in an effort to understand the role of the E. faecalis PP2C phosphatase (IreP) and Ser/Thr kinase (IreK) in mediating intrinsic cephalosporin resistance. Our results argue that the IreK/IreP kinase-phosphatase pair comprises the core of a signal transduction pathway that reciprocally regulates intrinsic cephalosporin resistance in E. faecalis. We used genetic analyses in two divergent lineages of E. faecalis—OG1 and T1, belonging to multilocus sequence types 1 and 21, respectively (31)—to show that mutants lacking the IreP phosphatase exhibit hyperresistance to cephalosporins. Given that the ireP ireK locus can be identified in all E. faecalis genomes sequenced to date (32–34), it seems likely that the IreP/IreK signaling system reciprocally controls cephalosporin resistance in most (or all) isolates of E. faecalis. Additionally, our genetic analysis indicates that phosphorylation of IreK leads to kinase activation in vivo (Table 2) and further suggests that a critical biological role of the IreP phosphatase is to regulate IreK activity—and, by extension, cephalosporin resistance—by controlling the level of IreK phosphorylation. The observation that ireK deletion is  

In the presence of cephalosporin stress, the inhibitory (phosphatase) activity of IreP on IreK would be counterproductive to E. faecalis, as enhanced kinase activity is desirable to promote resistance. Therefore, we reasoned that a mechanism might exist to transiently overcome the negative regulatory effect of IreP—to attenuate the phosphatase activity of IreK—upon kinase activation. Our results suggest that enhanced IreK kinase activity may indeed lead to a reduction in IreP phosphatase activity (Fig. 4), in principle enabling the kinase pool to become (at least transiently) more active and mediate signaling to upregulate the as-yet-unknown resistance mechanism(s). While more work is needed to unravel the mechanism underlying this observation, we speculate that IreK may phosphorylate IreP directly to inhibit its phosphatase activity. We hypothesize that with IreP in an “inactive” state, a pool of IreK could become more highly phosphorylated (activated) and serve to activate downstream cephalosporin resistance mechanisms. A recent report suggested that the IreP homolog of M. tuberculosis (PstP) can be phosphorylated by its cognate kinase (35), although in that case phosphorylation appeared to activate, rather than inhibit, phosphatase activity. We tested to see if recombiant E. faecalis IreK-n could phosphorylate IreP in vitro but were unable to observe any evidence of phosphorylation under our conditions (data not shown).

The observation that the ΔireP mutant exhibited hyperresistance to cephalosporins but not to ampicillin (Table 1) is intriguing given that both cephalosporins and ampicillin belong to the β-lactam class of antibiotics, all of which are thought to exert their antimicrobial activity via inactivation of PBPs to prevent PG cross-linking. We speculate that the inherent differences in affinity for PBPs of broad-spectrum cephalosporins compared to ampicillin account for this disparity. Ampicillin presumably inhibits the entire repertoire of enterococcal PBPs efficiently (leading to relatively low MICs), whereas the cephalosporins are unable to bind efficiently to Pbp5 and cannot inhibit its activity (leading to intrinsic cephalosporin resistance). Exposure to cephalosporins may therefore lead to a unique physiological state in which Pbp5 is active—but other PBPs are inhibited—that somehow triggers IreK activation. The PASTA domains of IreK-like kinases appear to bind PG or fragments thereof (10–12), suggesting that cephalosporin exposure could lead to accumulation of an IreK-activating ligand in PG as a component of this mechanism.
However, the CroRS two-component signaling system is also required for full cephalosporin resistance in *E. faecalis* (7, 8), indicating that the resistance signaling pathway is more complex and may involve additional signals. The mechanism by which IreK/P is integrated with CroRS to confer resistance is unknown, but IreK-like kinases from streptococci are known to phosphorylate two-component response regulators (36, 37), suggesting a potential route for direct control of the CroR response regulator by IreK. Our initial attempts to phosphorylate CroR with IreK route for direct control of the CroR response regulator by IreK. component response regulators (36, 37), suggesting a potential integrated with CroRS to confer resistance is unknown, but IreK-may involve additional signals. The mechanism by which IreK/P is indicating that the resistance signaling pathway is more complex and may involve additional signals. The mechanism by which IreK/P is integrated with CroRS to confer resistance is unknown, but IreK-like kinases from streptococci are known to phosphorylate two-component response regulators (36, 37), suggesting a potential route for direct control of the CroR response regulator by IreK. Our initial attempts to phosphorylate CroR with IreK in vitro have proven unsuccessful (data not shown). Future studies will address the functional interconnections between these signaling systems.

Many studies have shown that antibiotic-resistant bacteria are less fit than their susceptible counterparts in the absence of antibiotic stress (38). For example, a recent study demonstrated that constitutive expression of vancomycin resistance in enterococci leads to a significant fitness reduction in the absence of vancomycin (39) and, furthermore, that tight regulation of vancomycin resistance expression by the VanSR signal transduction system reduces the biological cost of vancomycin resistance dramatically. Our results indicate that constitutive cephalosporin resistance also imposes a substantial fitness cost on *E. faecalis* in the absence of cephalosporins (Fig. 5). The molecular basis for this fitness cost is not yet clear, in part because the output of the IreK/IreP signaling pathway is unknown. In any case, regulation of cephalosporin resistance by the IreK/IreP signaling system plays a critical role in balancing the expression of resistance functions given the needs of the cell in a particular environment to minimize the biological cost associated with resistance and maximize the ability of *E. faecalis* to be competitive in the face of environmental fluctuations it encounters in the GI tract.

**TABLE 3 Strains and plasmids used in this study**

| Strains or plasmid       | Relevant description or genotypea | Source or reference |
|--------------------------|----------------------------------|---------------------|
| **Strains**              |                                  |                     |
| *E. coli*               |                                  |                     |
| TOP10                    | Routine cloning host             | Invitrogen          |
| BL21(DE3)                | Protein overproduction host      | Lab stock           |
| *E. faecalis*           |                                  |                     |
| OG1                      | Wild-type, original unmarked isolate (MLST 1) | 43                   |
| OG1RF                    | Spontaneous rifampin-resistant and Fα derivative of OG1 | 44                   |
| CK119                    | OG1RF ΔireK2                     | This work           |
| CK121                    | OG1RF ΔireP2                     | This work           |
| CK125                    | OG1RF Δ(ireP-ireK)2              | This work           |
| OG1Sp                    | Spontaneous Fp derivative of OG1  | 40                   |
| JL178                    | OG1Sp ΔireP2                     | This work           |
| CK138                    | Spontaneous Fα derivative of OG1 | This work           |
| CK204                    | CK138 ΔireP2                     | This work           |
| T1 (SS498)               | Wild-type (MLST 21), CDC reference strain | 45                   |
| IL202                    | T1 ΔireK2                        | This work           |
| IL204                    | T1 ΔireP2                        | This work           |
| **Plasmids**            |                                  |                     |
| pCJK47                   | Counterselectable vector for allelic exchange | 40                   |
| pCJK74                   | ΔireK2 allele in pCJK47           | This work           |
| pCJK75                   | ΔireP2 allele in pCJK74          | This work           |
| pCJK105                  | Δ(ireP-ireK)2 allele in pCJK47    | This work           |
| pCJK111                  | pET28b::ireK-n (kinase/juxtamembrane domain) | This work |
| pCJK112                  | pET28b::ireP                     | This work           |
| plRG8                    | *E. faecalis* expression vector, constitutive P23 promoter (Em) | This work |
| pJL25                    | pJRG8::ireP                      | This work           |
| pCJK160                  | pJRG8::ireP+ireK                 | This work           |
| pCJK201                  | pJRG8::ireP ireK(T163/166/168E)   | This work           |
| pCJK216                  | pJRG8::ireP ireK K41R            | This work           |

a MLST, multilocus sequence type.

**MATERIALS AND METHODS**

**Bacterial strains, growth media, and chemicals.** Strains used in this study are listed in Table 3. Brain heart infusion medium (BHI) and Mueller-Hinton broth (MHB) were prepared as described by the manufacturer (Becton Dickinson). Bacteria were stored at −80°C in BHI supplemented with 30% glycerol. Antibiotics and other chemicals were obtained from Sigma unless otherwise indicated. Erythromycin (Em) was used at 10 μg/ml, spectinomycin (Sp) at 1,000 μg/ml, and fusidic acid (Fa) at 25 μg/ml for growth of resistant *E. faecalis*.

**Construction of *E. faecalis* mutants.** All PCR amplifications used *E. faecalis* OG1RF genomic DNA as the template and *Pfu* II Ultra polymerase (Stratagene). The markerless exchange system described by Kristich et al. (40) was used to construct unmarked, in-frame deletions of ireP in various genetic backgrounds. Briefly, a derivative of plasmid pCJK47 carrying an in-frame deletion allele of ireP (pCJK75) was constructed using the Bsal-based cloning scheme (40) to seamlessly fuse two PCR amplicons flanking ireP to form the in-frame deletion. The deletion allele was designed such that the first 10 codons and the last 10 codons of the ireP gene remained, in an effort to avoid any unanticipated effects on expression of adjacent genes, removing 92% of the ireP gene. This ΔireP allele was transferred to the native ireP location in the *E. faecalis* chromosome using pVE6007 as a helper plasmid to facilitate recombination as previously described (41). Successful isolation of ΔireP mutants was achieved after incubation of counterselection plates at room temperature or 30°C for ∼3 days. An *E. faecalis* double mutant lacking ire and ireK (CK125) was constructed via an analogous strategy, using a pCJK47 derivative (pCJK105) carrying an in-frame deletion of both ireP and ireK. This double mutant allele retained the first 10 codons of ireP and the last 6 codons of ireK. Finally, the previously described ΔireK2 allele was introduced into *E. faecalis* T1 using pVE6007-assisted recombination of pCJK74.
Construction of plasmids. A plasmid to express genes in *E. faecalis* was constructed by amplifying the constitutive $P_{\text{Tn}}$ promoter (lacking the last 34 nucleotides) from pDL278p23 (42) and cloning it with primer-specified BglIII and SpeI restriction sites to replace the rhamnose-inducible promoter of pJCK96 (9), creating pIRG8. For complementation of the $\Delta$ireP mutation, $\text{ireP}$ was amplified and cloned into pIRG8 using primer-specified SpH1 and XhoI sites, yielding pIL25. For analysis of mutant $\text{ireK}$ alleles, we chose to express both $\text{ireP}$ and $\text{ireK}$ from pIRG8 (introduced into an *E. faecalis* host lacking both genes, CK125) due to the likely translational coupling of these genes, in an attempt to ensure that both gene products were produced in the appropriate stoichiometry reflecting the natural state. To do so, the locus containing $\text{ireP}$ and $\text{ireK}$ was amplified and cloned using primer-specified SpH1/XhoI sites into pIRG8, creating pCKJ160. We note that, for unrelated purposes, an artificial XbaI site was introduced near the end corresponding to the C terminus of IreP.

Antibiotic susceptibility. MICs of antibiotics were determined in aerobic liquid cultures using a microtiter plate serial dilution method in a Bioscreen C plate reader (Oy Growth Curves Ab, Ltd.). Twofold dilutions of antibiotics in MHB were prepared in the wells of a 100-well honeycomb microtiter plate. Bacteria from stationary-phase cultures in MHB were inoculated into each well to a concentration of $10^8$ CFU/ml. Plates were incubated at 37°C for 24 h with brief shaking and measurement of optical density at 600 nm ($\text{OD}_{600}$) at 15-min intervals. The lowest concentration of antibiotic that prevented growth was recorded as the MIC. In some cases, antibiotic susceptibility was also assessed by preparing serial 10-fold dilutions of stationary-phase cultures and inoculating aliquots onto the surface of agar plates supplemented with antibiotics.

Protein purification. Overnight cultures of *E. coli* BL21(DE3) carrying the desired expression plasmid were diluted 50-fold in fresh LB media and cultured at 37°C for 3 h. Cells were induced with 1 mM IPTG (isopropyl-$\beta$-d-thiogalactopyranoside) for 1 h and collected by centrifugation, suspended in 10 ml of binding buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 5 mM imidazole), and treated with 1 mg/ml lysozyme for 20 min at 37°C. After disruption by sonication, the lysates were clarified by centrifugation (35,000 $\times$ g for 15 min) and passed through a 0.2-$\mu$m filter. Clarified lysates were applied to Profinity Ni-charged resin (Bio-Rad) pre-equilibrated with binding buffer and washed with 10 column volumes of wash buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 20 mM imidazole), and bound proteins were recovered with elution buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 500 mM imidazole). Fractions containing the protein of interest were pooled and dialyzed into storage buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol) at 4°C.

Kinase activity of IreK-n. Purified 6His-IreK-n (1.3 $\mu$M) was incubated with myelin basic protein (9.4 $\mu$M) in kinase buffer (50 mM Tris [pH 7.5], 25 mM NaCl, 1 mM MnCl$_2$, 1 mM MgCl$_2$, 1 mM dithiothreitol, 0.1 mM EDTA) at 37°C in the presence or absence of ATP (2 mM). In some cases, purified 6His-IreP was included at 0.8 $\mu$M. Aliquots were removed at intervals and quenched with Laemmli sodium dodecyl sulfate (SDS) sample buffer. Following SDS-polyacrylamide gel electrophoresis (PAGE), ProQ Diamond phosphoprotein stain (Invitrogen) was used to detect phosphorylated proteins according to the manufacturer’s instructions. Total protein was subsequently detected in the same gel using GelCode blue (Pierce).

Phosphatase activity of purified IreP. Reactions were carried out in volumes of 100 $\mu$l in 96-well plates. Purified 6His-IreP (168 $\mu$M) was incubated with 20 mM para-nitrophenyl phosphate (Pierce) in 50 mM Tris (pH 8.0) supplemented with various concentrations of MnCl$_2$ or MgCl$_2$ for 20 min at room temperature. Reactions were terminated by addition of 50 $\mu$l of 2 M NaOH, and absorbance at 405 nm was measured.

Phosphatase activity in lysates. Cultures growing exponentially in BHI plus Em were harvested by centrifugation and stored at $-20^\circ$C. thawed pellets were washed three times with ultrapure water and suspended in 250 $\mu$l of lysis buffer (50 mM Tris [pH 7.4], 1 mM EGTA, 0.2% Triton X-100, 0.1% $\beta$-mercaptoethanol) containing 1× HALT protease inhibitor (Pierce). Bacteria were disrupted by bead beating. Beads and intact bacteria were collected by centrifugation (16,000 $\times$ g, 15 min), and lysates were passed through desalting columns (Bio-Spin 6, Bio-Rad). Protein concentration was determined using Coomassie plus protein assay reagent (Pierce). Phosphatase activity was determined with a serine/threonine phosphatase assay system kit (Promega). Desalted lysates were added at a final concentration of 10 $\mu$g/ml to reaction mixtures containing 50 mM imidazole (pH 7.2), 5 mM MnCl$_2$, 0.02% $\beta$-mercaptoethanol, 200 $\mu$M EGTA, and 200 $\mu$M phosphothreonine peptide and incubated at 37°C. Control reaction mixtures did not contain phosphopeptide. At intervals, aliquots were removed and mixed with an equal volume of mobility dye substrate to stop the reaction. Absorbance was measured at 630 nm.

Competition experiments. Strains to be competed were cultured (separately) overnight in MHB. Culture density ($\text{OD}_{600}$) was determined, and the differentially marked wild-type and mutant strains were mixed in the desired proportion (typically ~90 to 95% mutant, 5 to 10% wild-type) in fresh MHB. The mixtures were subjected to serial dilutions, and aliquots were plated on BHI agar plates supplemented with appropriate antibiotics to enumerate wild-type and mutant cells in the inoculum. The mixed inoculum was diluted to a density of $10^8$ CFU/ml and incubated at 37°C until the following day. Dilutions and enumeration were repeated on successive days.

Results and immunoblots. Anti-IreK antisera (a generous gift from Patrick Schlievert) was produced by immunization of Dutch Belted rabbits with purified 6His-IreK-n and used at a dilution of 1:10,000 for immunoblots with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen). *E. faecalis* sigma factor ($\sigma^h$) was detected using anti-RNA polymerase sigma 70 monoclonal antibody 2G10 (Abcam) with goat anti-mouse IgG HRP-conjugated secondary antibodies (Invitrogen). Total cell lysates of *E. faecalis* strains for immunoblot analysis were prepared from exponentially growing cells by digestion with 5 mg/ml lysozyme in lysozyme buffer (20 mM Tris [pH 8.0], 10 mM EDTA) for 20 min at 37°C prior to solubilization in Laemmli SDS sample buffer.

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