Reciprocal Regulation of PASTA Kinase Signaling by Differential Modification

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ABSTRACT Transmembrane Ser/Thr kinases containing extracellular PASTA (penicillin-binding protein [PBP] and Ser/Thr-associated) domains are ubiquitous among Actinobacteria and Firmicutes species. Such PASTA kinases regulate critical bacterial processes, including antibiotic resistance, cell division, cell envelope homeostasis, and virulence, and are sometimes essential for viability. Previous studies of purified PASTA kinase fragments revealed they are capable of autophosphorylation in vitro, typically at multiple sites on the kinase domain. Autophosphorylation of a specific structural element of the kinase known as the activation loop is thought to enhance kinase activity in response to stimuli. However, the role of kinase phosphorylation at other sites is largely unknown. Moreover, the mechanisms by which PASTA kinases are deactivated once their stimulus has diminished are poorly understood. Enterococcus faecalis is a Gram-positive intestinal bacterium and a major antibiotic-resistant opportunistic pathogen. In E. faecalis, the PASTA kinase IreK drives intrinsic resistance to cell wall-active antimicrobials, and such antimicrobials trigger enhanced phosphorylation of IreK in vivo. Here we identify multiple sites of phosphorylation on IreK and evaluate their function in vivo and in vitro. While phosphorylation of the IreK activation loop is required for kinase activity, we found that phosphorylation at a site distinct from the activation loop reciprocally modulates IreK activity in vivo, leading to diminished activity (and diminished antimicrobial resistance). Moreover, this site is important for deactivation of IreK in vivo upon removal of an activating stimulus. Our results are consistent with a model in which phosphorylation of IreK at distinct sites reciprocally regulates IreK activity in vivo to promote adaptation to cell wall stresses.

IMPORTANCE Transmembrane Ser/Thr kinases containing extracellular PASTA domains are ubiquitous among Actinobacteria and Firmicutes species and regulate critical processes, including antibiotic resistance, cell division, and cell envelope homeostasis. Previous studies of PASTA kinase fragments revealed autophosphorylation at multiple sites. However, the functional role of autophosphorylation and the relative impacts of phosphorylation at distinct sites are poorly understood. The PASTA kinase of Enterococcus faecalis, IreK, regulates intrinsic resistance to antimicrobials. Here we identify multiple sites of phosphorylation on IreK and show that modification of IreK at distinct sites reciprocally regulates IreK activity and antimicrobial resistance in vivo. Thus, these results provide new insights into the mechanisms by which PASTA kinases can regulate critical physiological processes in a wide variety of bacterial species.

KEYWORDS PASTA kinase, antimicrobial resistance, cell wall stress, regulatory phosphorylation

Enterococci are members of the human commensal gut microbiota (1). These Gram-positive bacteria (primarily Enterococcus faecalis and Enterococcus faecium in humans) are major causes of opportunistic infections, especially in hospital settings (2, 3).
Enterococci are intrinsically resistant to a wide variety of environmental stressors, including several antibiotics such as the cephalosporin class β-lactams (4). This intrinsic cephalosporin resistance enables enterococci to proliferate to high levels of abundance in the intestine during cephalosporin treatment and subsequently to translocate to other locations in the body, where they can cause diseases such as endocarditis and bacteremia (5–8). In fact, treatment with cephalosporins is a known risk factor for subsequent enterococcal infection (9, 10). Understanding the mechanisms of intrinsic cephalosporin resistance may enable identification of new therapeutic targets or strategies to prevent or treat enterococcal infections.

Ongoing research has revealed several cephalosporin resistance determinants in enterococci. One in particular, a transmembrane eukaryotic-type Ser/Thr kinase (eSTK) known as IreK in *E. faecalis* (formerly called PrkC (11, 12)) or Stk in *E. faecium*, is essential for resistance (13, 14). These eSTKs are members of a superfamily of kinases present in all domains of life, referred to as the Hanks family, defined by a common catalytic core which phosphorylates target serine, threonine, or tyrosine residues (15). IreK regulates cephalosporin resistance in a manner directly dependent on its kinase activity (12, 16), although the effectors downstream of IreK in the resistance pathway are not fully understood. One IreK substrate is IreB, a protein which is phosphorylated by IreK directly in vitro and is inhibitory to cephalosporin resistance via an unknown mechanism (17).

*E. faecalis* mutants lacking IreK, or carrying a catalytically impaired IreK variant, are drastically impaired in their cephalosporin resistance. Conversely, *E. faecalis* mutants lacking the cognate phosphatase (IreP), in which IreK is constitutively phosphorylated and active, exhibit elevated levels of resistance (12, 16). Every environmental condition tested thus far that was found to enhance IreK autophosphorylation also resulted in enhanced IreK-dependent IreB phosphorylation in vivo (16), indicating that phosphorylation can lead to activation of IreK. IreK phosphorylation, IreB phosphorylation, and cephalosporin resistance appear to be directly correlated (16).

IreK belongs to a subset of the eSTKs that exhibit a characteristic domain architecture, all of which include 3 to 5 extracellular PASTA (penicillin-binding protein [PBP] and Ser/Thr-associated) domains. The functions of PASTA domains are poorly understood, although binding to peptidoglycan or fragments thereof has been proposed (18–21). These PASTA-containing “PASTA kinases” are ubiquitous in *Actinobacteria* and *Firmicutes* and regulate a wide variety of functions, including cell division, virulence, antibiotic resistance, and toxin production (22–28). In some organisms, the PASTA kinase is essential for viability (23, 29). Despite their importance, many questions about the molecular mechanisms by which PASTA kinases transduce signals remain.

A model for PASTA kinase signaling has been proposed (30, 31) in which ligand binding leads to homodimerization, enabling autophosphorylation of a centrally located structural element known as the activation loop. Phosphorylation of the activation loop is thought to produce a conformational change that activates the kinases, enabling enhanced phosphorylation of downstream substrates. Deactivation of PASTA kinases once their output is no longer needed is poorly understood but presumably requires the action of their cognate phosphatases to dephosphorylate the kinase activation loop to return the kinase to its “off” state. The regulatory inputs that control the in vivo dynamics of these competing processes—kinase-mediated loop phosphorylation versus phosphatase-mediated dephosphorylation—are not understood. How, or if, the activity of the phosphatases themselves is regulated also remains unknown.

The kinase domain of PASTA kinases adopts the characteristic two-lobed structure of eukaryotic Ser/Thr kinases, comprised of a mixed alpha/beta N-terminal lobe and a helical C-terminal lobe (32–34). The activation loop is centrally located near the interface between the lobes and the entrance to the active site but has not been resolved in PASTA kinase crystal structures, suggesting conformational flexibility. In vitro studies performed with the purified kinase domains of PASTA kinases have revealed phosphorylation at sites in addition to the activation loop. For example, phosphorylation of the “juxtamembrane” segment that connects the C-terminal lobe of the kinase domain to the transmembrane helix has been consistently identified for
different PASTA kinases (32, 35–37). In most cases, the function of phosphorylation at these sites is not known, although phosphorylation of one juxtamembrane site on PknB from *Mycobacterium tuberculosis* influences the affinity of a substrate (FhaA) for the kinase (38). Phosphorylation at another site in the helical lobe of the kinase catalytic core has also been observed for the PASTA kinases from *Bacillus subtilis* (35), *Bacillus anthracis* (39), and *Listeria monocytogenes* (40). Alanine substitution variants at this site of the IreK homologs in *B. subtilis* and *B. anthracis* (S214) resulted in reduced kinase activity in vitro, but the effects of modification at this site in vivo were not reported.

Here we identify multiple sites of phosphorylation on IreK and evaluate their function in vivo and in vitro. As expected, phosphorylation of the IreK activation loop at multiple sites is required for kinase activity. However, phosphorylation at a site in the helical lobe of the IreK catalytic core reciprocally modulates IreK activity in vivo, leading to diminished activity (and diminished antimicrobial resistance). Moreover, this site is important for deactivation of IreK in vivo upon removal of an “activating” stimulus. Our results are consistent with a model in which phosphorylation of IreK at distinct sites reciprocally regulates IreK activity in vivo to promote adaptation to cell wall stresses.

**RESULTS**

**IreK is phosphorylated at multiple sites.** To identify sites of phosphorylation on IreK, we purified a recombinant form of the cytoplasmic IreK kinase domain (IreK-n [12]) and incubated it with ATP in vitro for autophosphorylation to occur (17). The resulting protein was analyzed by mass spectrometry, revealing seven potential sites of phosphorylation (Fig. 1; see also Fig. S1 in the supplemental material). Most of the identified sites were found at locations in the IreK protein analogous to phosphorylation sites previously identified for other PASTA kinases, including on the activation loop (T163, T166, and T168), in the juxtamembrane segment (T285 and T291), and at a site in the helical lobe of the kinase structure (T218). Phosphorylatable residues (Thr or Ser) are well conserved at corresponding positions of PASTA kinases from a diverse collection of bacteria, consistent with the hypothesis that phosphorylation at these positions represent evolutionarily conserved regulatory mechanisms (Fig. S1). One site (T148)
found in the kinase catalytic core does not correspond to any previously identified sites of phosphorylation from other PASTA kinases, and phosphorylatable residues are not conserved at this position of diverse PASTA kinases (Fig. S1). Inspection of the local sequences surrounding the 7 phosphorylation sites did not reveal any obvious consensus recognition motif that would define a phosphorylation site (Fig. S1).

To assess the physiological relevance of phosphorylation at these sites, we constructed a series of *E. faecalis* mutants with substitutions of either alanine (which is nonphosphorylatable) or glutamate (which often acts as a phosphomimetic) codons in the chromosomal *ireK* gene. Because IreK drives cephalosporin resistance (11, 12, 16), we determined the level of resistance to ceftriaxone (a broad-spectrum cephalosporin) for the panel of mutants (Table 1). Substitutions at T148, T285, and T291, alone or in combinations, did not lead to substantial changes in the extent of cephalosporin resistance compared to the otherwise isogenic wild-type strain, suggesting either that these sites are not phosphorylated *in vivo* or that their phosphorylation is not functionally important for IreK to regulate cephalosporin resistance.

In contrast, substitutions at the other identified sites led to marked changes in ceftriaxone resistance. We previously analyzed a mutant in which residues at all 3 of the predicted sites of phosphorylation on the IreK activation loop (T163, T166, and T168) had been replaced collectively. The resulting triple-alanine IreK mutant was unable to become activated or to mediate cephalosporin resistance (16), but it was not clear if all 3 predicted sites—or only a subset—were functionally important. Our mass spectrometry results confirmed that all 3 predicted sites on the activation loop could indeed be phosphorylated. Analyses of our panel of single- and double-substitution mutants revealed that each activation loop single- or double-alanine mutant exhibited a reduction in ceftriaxone resistance compared to the otherwise isogenic wild-type strain, suggesting either that these sites are not phosphorylated *in vivo* or that their phosphorylation is not functionally important for IreK to regulate cephalosporin resistance.

Alanine or glutamate substitutions at T218 resulted in robust and reciprocal changes in ceftriaxone resistance: the T218A mutant exhibited elevated resistance, while the T218E mutant exhibited reduced resistance compared to the wild-type strain (Table 1). The reciprocal relationship of phosphoablative and phosphomimetic mutations sug-

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**TABLE 1** Ceftriaxone resistance of *E. faecalis* strains

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Ceftriaxone MICb (μg/ml)</th>
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<tr>
<td>OG1 (wild type)</td>
<td>64</td>
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<tr>
<td>ΔireK</td>
<td>1</td>
</tr>
<tr>
<td>ireK T163A T166A T168Ac</td>
<td>2</td>
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<tr>
<td>ireK T163E T166E T168Ec</td>
<td>64</td>
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<tr>
<td>ireK T163A</td>
<td>8</td>
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<td>ireK T166A</td>
<td>16</td>
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<td>ireK T168A</td>
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<td>ireK T166A 168A</td>
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<tr>
<td>ireK T163A T168A</td>
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<tr>
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<td>8</td>
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<td>16</td>
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<td>ireK T285A T291A</td>
<td>32</td>
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**a**All strains other than the wild-type strain are isogenic derivatives of OG1.

**b**Median MIC obtained from three biological replicates following 24 h of incubation in MHB at 37°C.

**c**Data are from reference 16.
suggests that T218 represents an authentic site of phosphorylation-dependent IreK regulation and, moreover, that phosphorylation of T218—in contrast to phosphorylation of the activation loop—serves to downregulate IreK activity and cephalosporin resistance. To test this hypothesis, we analyzed phosphorylation of the IreK T218 substitution variants \textit{in vivo} using Phos-tag SDS-PAGE, as we had previously (16). During Phos-tag SDS-PAGE, phosphorylated protein proteoforms migrate more slowly than unphosphorylated proteoforms, enabling separation and quantitation of unphosphorylated versus phosphorylated proteoforms (lower bands versus middle and upper bands, respectively, in Fig. 2). Of note, the most slowly migrating IreK proteoform was absent from both mutants in which T218 cannot be phosphorylated, suggesting that T218 phosphorylation is required for generation of this IreK proteoform. By extension, the bands in the middle region likely represent IreK proteoforms phosphorylated on the activation loop (T163, T166, and T168), although we cannot rigorously assign specific phosphorylated proteoforms to specific bands at this time. Regardless, reciprocal changes in overall IreK phosphorylation (presumably on the activation loop) were observed upon substitution of T218. In cells growing exponentially either with or without exposure to ceftriaxone stress, the T218A mutant exhibited an increased level of IreK phosphorylation (despite the absence of one phosphorylation site) compared to wild-type IreK (see middle bands in Fig. 2). Conversely, the T218E mutant exhibited reduced levels of IreK phosphorylation, either with or without ceftriaxone stress. Hence, the level of phosphorylation of IreK (and therefore the level of IreK activity [16]) \textit{in vivo} is modulated in a reciprocal manner by phosphoablative or phosphomimetic substitutions at T218, mimicking the phenotypic effect on ceftriaxone resistance. This is consistent with our previous study, in which we observed that exposure to cell wall stressors promotes phosphorylation of IreK on the activation loop, which is required to enhance IreK activity \textit{in vivo} to produce elevated cephalosporin resistance (16). Hence, these results suggest that modification of T218 regulates IreK activity by influencing the extent of phosphorylation on the IreK activation loop.

This hypothesis predicts that substitutions affecting the activation loop should be dominant over substitutions at T218. To test this, we constructed combinatorial IreK mutants containing substitutions at both T218 and the activation loop (Table 1). Combining the activating T218A substitution with the inactivating triple-alanine activation loop substitutions (T163A, T166A, and T168A) abrogated the effect of the T218A
substitution and rendered IreK inactive and unable to promote ceftriaxone resistance (MIC of 2 \( \mu g/ml \), equivalent to that measured for the T163A T166A T168A mutant), consistent with the hypothesis. We also analyzed the effects of substitutions at T218 when residues at all three sites on the activation loop were replaced with glutamate (Table 1). In this scenario, IreK was active but the status of modification on the activation loop was “fixed.” Here, the effects on ceftriaxone resistance of alanine or glutamate substitutions at T218 were modest and markedly diminished (2-fold up or down) compared to the effects of T218 substitutions in the context of a wild-type (i.e., phosphorylatable) activation loop (8-fold up or down). Collectively, these results are consistent with the hypothesis that modification at T218 regulates IreK activity primarily by influencing the extent of phosphorylation on the IreK activation loop.

**IreK T218 mutants exhibit altered autophosphorylation in vitro.** To test if modification at T218 could directly influence the activity of purified, recombinant IreK, we examined autophosphorylation of IreK-n variants in vitro. Because IreK-n undergoes some autophosphorylation during overexpression in *Escherichia coli*, all recombinant proteins were dephosphorylated using the cognate phosphatase IreP prior to autophosphorylation assays. IreK-n exhibits a modest shift in electrophoretic mobility during standard SDS-PAGE that depends on its phosphorylation status (12), enabling us to quantify phosphorylation by measuring the relative amounts of IreK present in the distinct proteoforms. As a control to validate this mobility shift as a readout, simultaneous introduction of T163A T166A T168A substitutions (which prevent IreK phosphorylation and activity in vivo) in the IreK-n activation loop (yielding the “loop mutant”) resulted in a much slower accumulation of the upper phosphorylated proteoform that remained at less than 10% of the total IreK signal compared to the wild-type strain (Fig. 3). Introduction of the T218A substitution (which enhances IreK phosphorylation and activity in vivo) in IreK-n resulted in more-rapid accumulation of the phosphorylated IreK-n proteoform than was seen with the wild-type strain (Fig. 3), as expected if phosphorylation of T218 acts to impair IreK activity. Surprisingly, a similar result was observed upon introduction of the T218E substitution into IreK-n (Fig. 3). This observation is inconsistent with the in vivo results (i.e., that T218E results in reduced IreK activity) and suggests that other as-yet-unknown factors may be involved in T218-mediated regulation of IreK kinase activity in vivo.

One explanation for the disparate in vivo versus in vitro effects of the T218E substitution could be that IreK T218E recruits IreP (the cognate phosphatase) in vivo to promote dephosphorylation of IreK. To test that possibility, we performed IreP-mediated dephosphorylation reactions in vitro using recombinant prephosphorylated IreK-n variants as substrates. IreP dephosphorylated wild-type, T218A, and T218E variants of IreK-n with indistinguishable kinetics in vitro (Fig. S2), indicating that substitutions at T218 do not alter the ability of IreP to dephosphorylate IreK.

To test for an effect of substitutions at T218 on substrate phosphorylation, we performed in vitro phosphorylation reactions to assess IreK-n-mediated phosphorylation of a known substrate, IreB (17). IreK-n variants were maximally prephosphorylated and then added to recombinant IreB in vitro. The IreK-n loop mutant did not appreciably phosphorylate IreB in our reactions, as expected given the lack of in vivo phosphorylation and activity. In contrast, the wild-type, T218A, and T218E IreK-n variants all phosphorylated IreB with essentially indistinguishable kinetics (Fig. S3), suggesting that modification at T218 does not directly alter interactions with substrates. Together, the in vitro phosphorylation data indicate that the most likely mechanism by which modification at T218 impacts IreK function in vivo is by influencing IreK autophosphorylation, which is consistent with the genetic data presented above using combinatorial IreK substitution mutants.

**Physiological role of modification at T218.** The in vivo genetic data are consistent with a model in which phosphorylation of T218 acts to reduce phosphorylation of the IreK activation loop and hence to reduce IreK kinase activity. We hypothesized that phosphorylation of T218 could therefore represent a mechanism of negative feedback
to reduce IreK activity in circumstances where signaling was no longer needed. To test this, we evaluated IreK phosphorylation in response to changes in growth. We previously showed that IreK is stimulated in growing cells, leading to IreK phosphorylation, kinase activity, and phosphorylation of its substrate IreB. However, upon cessation of growth brought about by a variety of conditions—including suspension of cells in nutrient-poor environments (e.g., phosphate-buffered saline [PBS])—IreK is deactivated, reducing its overall phosphorylation and kinase activity, as well as phosphorylation of the substrate IreB, markedly (16). Thus, to test if modification of T218 is important for this reduction in IreK activity, we subjected growing cells to suspension in PBS and analyzed changes in IreK phosphorylation over time.

Analysis of the IreK T218A variant revealed that the reduction in overall IreK phosphorylation upon suspension in PBS was markedly impaired (Fig. 4B), exhibiting both slower kinetics of dephosphorylation and greater total IreK phosphorylation remaining after 5 min (~10% of total IreK in a phosphorylated state for the wild type compared with ~20% for T218A at 5 min postresuspension). This difference is especially prominent for the phosphorylated IreK species represented in the middle region, for which there was a statistically significant difference in abundance levels between the wild-type and T218A at multiple time points. Analysis of the T218E mutant (Fig. 4C) revealed the reciprocal trend: phosphorylation of IreK was reduced at the initial time point and rapidly decreased thereafter (although the results did not achieve statistical significance compared to the wild type at later time points). Together, these results are consistent with a model in which phosphorylation of T218 plays a key role in providing negative regulation of IreK kinase activity in vivo.

FIG 3 Effect of substitutions at IreK T218 on autophosphorylation of IreK in vitro. (A) Autophosphorylation reactions using dephosphorylated, recombinant IreK-n variants were performed at 37°C. Samples were quenched at intervals and subjected to SDS-PAGE and immunoblotting to detect IreK. (B) Signal intensities of the upper (phosphorylated) and lower (unphosphorylated) bands were compared from a minimum of 3 independent replicates to generate the graph. Error bars represent 1 standard deviation. The IreK-n variants were encoded by plasmids pCJK111 (wild type), pCLH50 (loop mutant; T163A T166A T168A), pCLH48 (T218A), and pCLH49 (T218E).
DISCUSSION

Transmembrane Ser/Thr kinases containing extracellular PASTA domains are ubiquitous among Actinobacteria and Firmicutes. Such PASTA kinases regulate critical bacterial processes, including antibiotic resistance, cell division, cell envelope homeostasis, and virulence, and are sometimes essential for viability. The PASTA kinase of E. faecalis, IreK, is critical for enterococcal intrinsic resistance to cephalosporins and colonization of the mammalian intestinal tract. We showed previously that phosphorylation of IreK is enhanced in response to cell wall stresses as well as in response to a signal associated with growth or cell division and that phosphorylation of IreK is required to enhance IreK kinase activity to promote cephalosporin resistance (16). However, the specific sites at which IreK becomes phosphorylated had not been comprehensively identified, nor had the functional consequences of phosphorylation at

FIG 4 Deactivation of the IreK T218A variant is impaired in vivo. Exponentially growing wild-type (A), IreK T218A (B), or IreK T218E (C) E. faecalis cells in MHB were rapidly filtered and subjected to resuspension in PBS to halt growth. Samples were harvested at intervals, and whole-cell lysates were analyzed by Phos-tag SDS-PAGE and immunoblotting for IreK. The signal intensities of the lower band (unphosphorylated proteoform) were compared with those of phosphorylated proteoforms (middle and upper bands) from a minimum of 3 independent biological replicates to generate the graphs (right). Asterisks indicate that differences between the wild-type and T218A strains in the phosphorylated proteoform in the middle band were statistically significant ($P < 0.05$) using Student’s t test (2 tailed, heteroscedastic). Error bars represent 1 standard deviation. Strains used were OG1 (wild type) and OK1 (T218A).
individual sites. Moreover, the mechanisms by which PASTA kinases, including IreK, are deactivated once their activity is no longer needed are poorly understood.

Here we identified seven sites at which IreK can become phosphorylated in vitro, using mass spectrometry to analyze the autophosphorylated cytoplasmic kinase domain of IreK. Most of the identified sites correspond to regions of the kinase domain which had been previously observed to be phosphorylated on other PASTA kinases. For example, phosphorylation in the juxtamembrane segment has been consistently observed for PASTA kinases, including PknB of *M. tuberculosis* and PrkC of *B. subtilis* (32, 35, 36). Phosphorylation of PknB in this region alters the binding affinity of a substrate protein (38). Mutant strains of *E. faecalis* producing IreK with substitutions at these sites did not exhibit substantial changes in the level of ceftriaxone resistance, suggesting that IreK phosphorylation in the juxtamembrane region is not functionally important for this response (or, alternatively, that these sites do not become phosphorylated in vivo). Similar findings were made for phosphorylation at T148 (which has not been observed for other PASTA kinases). Unlike the other sites identified, a phosphorylatable residue at the position corresponding to T148 is not widely conserved among diverse PASTA kinases, suggesting that T148 may not represent an authentic site of IreK phosphorylation in vivo.

We confirmed the prediction that the IreK activation loop can be phosphorylated, identifying three sites (T163, T166, and T168). Phosphorylation of the activation loop is a common feature of PASTA kinases and, indeed, of Ser/Thr kinases more broadly (41). Previous studies using purified PASTA kinase fragments revealed that phosphorylation of the activation loop is essential for kinase activity in vitro for both PknB and PrkC (35, 36, 39). We had previously shown that an IreK mutant with alanine substitutions at T163, T166, and T168 (which were predicted to be sites of phosphorylation based on inspection of the sequence) was not phosphorylated in vivo and was inactive, as it was unable to phosphorylate a known substrate in *E. faecalis* cells or promote ceftriaxone resistance (16). Here we positively confirmed that these predicted sites on the IreK activation loop can indeed become phosphorylated, and we evaluated the phenotypic effect of inactivating them individually. The results argue that phosphorylation at each of the three activation loop sites contributes to IreK activation in vivo, as each of the single mutants exhibits a defect in cephalosporin resistance. Phosphorylation at multiple activation loop sites appears to have an additive effect, as the triple alanine mutant is (4- to 8-fold) more susceptible than any of the single mutants. We cannot rule out the formal possibility that an alanine substitution per se at one site impacts the extent of phosphorylation at other activation loop sites, although if that were the case one might expect the single mutants to phenocopy the triple alanine mutant more closely. In any case, our data establish that phosphorylation of the activation loop in vivo is required for activation of the IreK kinase to drive cephalosporin resistance.

Phosphorylation at a site in the helical lobe of the kinase catalytic domain (S214 in the homologs from bacilli) has been observed previously for the PASTA kinases of *B. subtilis*, *B. anthracis*, and *L. monocytogenes* (35, 39, 40). S214A substitutions reduced the activity of recombinant kinase domain in vitro for the kinases of *B. subtilis* and *B. anthracis*, but the effects of S214 substitutions in vivo were not reported. Phosphorylation of serine was not identified in our data (despite using methods sufficient to detect phosphoserine), suggesting that IreK may exhibit an inherent preference for threonines or, alternatively, that serines become phosphorylated only under some experimental conditions not tested here. We identified phosphorylation of T218 on IreK, which is located in a similar region of the kinase catalytic domain structure as S214 of the *Bacillus* enzymes. However, alanine substitution at T218 led to enhanced, rather than reduced, IreK activity both in vitro and in vivo. Phosphoaablative or phosphomimetic substitutions at T218 had reciprocal effects on IreK activity in vivo, leading to enhanced (phosphoaablative) or reduced (phosphomimetic) IreK phosphorylation and ceftriaxone resistance. The influence of T218 modification on IreK activity requires that the IreK activation loop be available for phosphorylation, indicating that T218-mediated IreK regulation occurs primarily by modulating the ability of IreK to become phosphor-
ylated on its activation loop. Our results are consistent with a model in which phosphorylation of IreK at specific sites modulates its signaling activity in opposing ways: phosphorylation of sites on the activation loop enhances kinase activity, substrate (IreB) phosphorylation, and cephalosporin resistance, while phosphorylation of T218 diminishes overall IreK activity, IreB phosphorylation, and cephalosporin resistance by reducing activation loop phosphorylation. Thus, phosphorylation of T218 represents a form of negative feedback to keep overall levels of IreK activity in check and to enable the deactivation of IreK once the stimulatory stresses have been ameliorated. This phosphorylation event appears to be physiologically relevant, as the IreK T218A mutant does not become dephosphorylated (i.e., deactivated) as quickly or to the same extent as wild-type IreK upon loss of an activating stimulus. It is worth noting that a slowly migrating IreK proteoform (in the upper region of Fig. 4)—the production of which requires T218 to be phosphorylatable (Fig. 2)—appears to modestly increase in abundance immediately after the activating stimulus for IreK is removed (Fig. 4). Although this change was not found to be statistically significant under the conditions of our experiment, production of an IreK proteoform that is phosphorylated at T218 upon deactivation of IreK is wholly consistent with the model that phosphorylation of T218 provides negative feedback to reduce IreK activity. The biochemical mechanism by which phosphorylation at T218 impairs IreK activation loop phosphorylation remains unclear. One possibility is that phosphorylation of T218 helps to recruit (or activate) the cognate phosphatase IreP in vivo, thereby promoting IreK dephosphorylation and deactivation. If so, it seems likely that additional, as-yet-unidentified factors may also be required given that no changes in IreP phosphatase activity could be detected with purified components in vitro. Elucidating the mechanism by which modification of T218 influences IreK activity in vivo will be an important focus for future research.

**MATERIALS AND METHODS**

Detailed methods are provided in the supplemental material. Strains and plasmids used in this study are described in Table S1. Mueller-Hinton broth (MHB) was used for growth of *E. faecalis* in all experiments. Markerless allelic exchange was used to construct chromosomal mutations in the genome of *E. faecalis* OGI, as described previously (42, 43). Antibiotic resistance was determined by the broth microdilution method after 24 h at 37°C in 96-well plates. Phos-tag SDS-PAGE and immunoblotting and imaging were performed as described previously (16).

**IreK-n autophosphorylation.** Because recombinant His<sub>6</sub>-IreK-n variants are phosphorylated upon purification from *E. coli*, all variants were predephosphorylated by treatment with IreP prior to autophosphorylation reactions. For autophosphorylation, IreK-n variants were incubated in a reaction mixture of the following composition: 25 μM IreK-n, 1/3× Halt phosphatase inhibitor cocktail (Thermo Scientific) (3× concentration), 1 mM Ultra Pure ATP (Promega), and Mg<sup>2+</sup> buffer (40 mM Tris, 20 mM MgCl<sub>2</sub>, pH 7.5). Reactions were started by addition of IreK-n and quenched at intervals with SDS loading buffer.

**Dephosphorylation of IreK-n.** IreK-n variants were prephosphorylated as described above. Dephosphorylation reaction mixtures were as follows: 25 μM IreK-n, 0.1% β-mercaptoethanol, 1 μM IreP, and Mg<sup>2+</sup>/Mn<sup>2+</sup> buffer (50 mM Tris, 25 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.1 mM EDTA, pH 7.5). Reactions were started by addition of IreP and quenched at intervals with SDS loading buffer.

**Phosphorylation of IreB.** Reaction mixtures were as follows: 0.05 μM IreK-n, 1 μM Ultra Pure ATP (Promega), 25 μM IreB, and Mg<sup>2+</sup>/NaCl buffer (40 mM Tris, 150 mM NaCl, 20 mM MgCl<sub>2</sub>, pH 7.5). Reactions were initiated by the addition of IreK-n and quenched at intervals with SDS loading buffer.

**Identification of phosphorylation sites by mass spectrometry.** Recombinant IreK-n was allowed to autophosphorylate at 37°C to completion in the presence of excess ATP. After digestion with trypsin, mass spectral analysis was carried out on a nano-ultraperformance liquid chromatography (nano-UPLC) system (Waters Corporation) interfaced to a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA) with a nanoelectrospray ion source. The structural model of the IreK kinase domain was generated using I-TASSER (44, 45).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00016-19.

**SUPPLEMENTAL FILE 1**, PDF file, 1.3 MB.

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