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Structure and dimerization of IreB, a negative regulator of cephalosporin resistance in *Enterococcus faecalis*

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Abstract

Enterococcus faecalis, a leading cause of hospital-acquired infections, exhibits intrinsic resistance to most cephalosporins, which are antibiotics in the beta-lactam family that target cell-wall biosynthesis. A comprehensive understanding of the underlying genetic and biochemical mechanisms of cephalosporin resistance in *E. faecalis* is lacking. We previously determined that a transmembrane serine-threonine kinase (IreK) and its cognate phosphatase (IreP) reciprocally regulate cephalosporin resistance in *E. faecalis*, dependent on the kinase activity of IreK. Other than IreK itself, thus far the only known substrate for reversible phosphorylation by IreK and IreP is IreB, a small protein of unknown function that is well conserved in low-GC Gram-positive bacteria. We previously showed that IreB acts as a negative regulator of cephalosporin resistance in *E. faecalis*. However, the biochemical mechanism by which IreB modulates cephalosporin resistance remains unknown. As a next step towards an understanding of the mechanism by which IreB regulates resistance, we initiated a structure-function study on IreB. The NMR solution structure of IreB was determined, revealing that IreB adopts a unique fold and forms a dimer *in vitro*. Dimerization of IreB was confirmed *in vivo*. Substitutions at the dimer interface impaired IreB function and stability *in vivo*, indicating that dimerization is functionally important for the biological activity of IreB. Hence, these studies provide new insights into the structure and function of a widely conserved protein of unknown function that is an important regulator of antimicrobial resistance in *E. faecalis*.

Graphical abstract

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DUF965; PF06135; antimicrobial resistance; *Firmicutes*; domain-swapped

Introduction

The Gram-positive bacterium *Enterococcus faecalis* is a leading cause of hospital-acquired infections^{1; 2; 3}. *E. faecalis* is intrinsically resistant to cephalosporins, and prior cephalosporin therapy is a well-known risk factor for the acquisition of enterococcal infections^{4; 5; 6}. Cephalosporins are a sub-class of the beta-lactam family of antibiotics that impair the final stages of peptidoglycan synthesis by inhibiting the D, D-transpeptidase activity of peptidoglycan biosynthetic proteins, known as penicillin binding proteins, which cross-link peptidoglycan to maintain cell wall integrity. Although the genetic and biochemical mechanisms of cephalosporin resistance in *E. faecalis* are still being unraveled, recent studies have revealed a critical role for the signal transduction system comprised of a eukaryotic-like serine/threonine kinase (IreK) and its cognate phosphatase (IreP) that antagonistically regulate cephalosporin resistance^{7; 8}.

IreK is a transmembrane serine/threonine kinase that has been hypothesized to sense cephalosporin-induced cell wall damage, activate its kinase activity through autophosphorylation, and initiate a signaling pathway as part of an adaptive biological response that promotes cephalosporin resistance. IreP is thought to regulate the IreK signaling pathway by dephosphorylating IreK to keep kinase activity in check, as well as by dephosphorylating downstream substrates of IreK, presumably when the stimulatory cell wall damage has been repaired. However, little is known about the direct substrates that are phosphorylated by IreK, or about other downstream effectors in the IreK pathway that drive resistance. We identified one *E. faecalis* protein, IreB, that is reversibly phosphorylated by the IreK kinase and IreP phosphatase at residues Thr4 and Thr7⁹.

IreB is a cytosolic 10.5 kDa protein of unknown function that is highly conserved among low-GC Gram-positive bacteria (as are homologs of IreK). Bioinformatic analyses of IreB

provides no clues about its function, as its only identifiable domain is a “domain of unknown function” (DUF965; PF06135) spanning nearly the entire length of the protein. DUF965 domains are essentially found only in homologs of IreB in other Gram-positive bacteria (which have not been functionally characterized). Our studies of *E. faecalis* mutants lacking *ireB* revealed that IreB acts downstream of IreK as a negative regulator of cephalosporin resistance in *E. faecalis*⁹, although the precise biochemical function of IreB remains unknown. IreB can be modified by phosphorylation *in vivo*, and mutations that prevent modification at the known sites of phosphorylation (T4 and T7) influence the ability of IreB to modulate cephalosporin resistance⁹. Intriguingly, two-dimensional PAGE studies of an IreB mutant that cannot be phosphorylated at Thr4 or Thr7 revealed 2 isoforms with distinct isoelectric points, suggesting that IreB may be subject to modification at an additional as-yet-unknown site *in vivo*⁹. Although these findings collectively shed some light on the *in vivo* role of IreB in *E. faecalis*, the biochemical mechanism by which IreB exerts its effect on cephalosporin resistance remains unknown.

As a next step towards an understanding of the mechanism by which IreB negatively regulates enterococcal cephalosporin resistance, we initiated a structure-function study on IreB. The NMR solution structure of IreB was determined, revealing that IreB adopts a unique fold and forms a dimer *in vitro*. Dimerization of IreB was confirmed *in vivo*. Substitutions at the dimer interface impaired IreB function and stability *in vivo*, indicating that dimerization is functionally important for the biological activity of IreB. Hence, these studies provide new insights into the structure and function of a widely conserved protein of unknown function that is an important regulator of antimicrobial resistance in *E. faecalis*.

Results

NMR structure of IreB

To begin understanding the mechanisms by which IreB regulates cephalosporin resistance in *E. faecalis*, we used NMR to elucidate the IreB solution structure. IreB was determined to be a homodimer using specialized NMR techniques to distinguish intermonomer from intramonomer contacts¹⁰. A differentially labeled sample was prepared by mixing unlabeled protein with uniformly ¹⁵N/¹³C labeled protein. The 3D F1-¹³C -filtered, F3-¹³C -edited NOESY data enabled the assignment of 138 intermolecular NOEs that defined the orientation of the monomers relative to one another. The final ensemble of 20 conformers is shown in Figure 1a and the structural statistics are presented in Table 1. Backbone RMSD values of 0.53/0.58 Å and 0.68 Å were determined for the individual monomers and the dimer, respectively, using a residue range of 18–89. The close agreement of the backbone RMSD values between the monomers and the dimer indicates that the dimer interface is highly determined allowing for the proper orientation of the two halves. No long range NOEs were observed for residues 1–17, and ¹⁵N-¹H heteronuclear NOE values (Fig. 1b) confirmed that those N-terminal residues are dynamically disordered on the picosecond to nanosecond timescale. This N-terminal segment contains the known sites of phosphorylation (T4 and T7), and flexibility in this region likely facilitates entry into the catalytic cleft of the cognate IreK kinase for phosphorylation to occur.

Each IreB monomer is comprised of four α -helices. Helices $\alpha 1$ and $\alpha 2$ and helices $\alpha 3$ and $\alpha 4$ are connected by short 4–5 residue loops, while helices $\alpha 2$ and $\alpha 3$ are separated by an 11-residue loop containing a single turn of 3^{10} helix. Helix $\alpha 4$ from each monomer plays a critical role in intersubunit interactions, adopting a partially domain-swapped conformation (Fig. 1c). These helices are oriented at a 68° angle relative to each other and form the core of the dimer interface through hydrophobic interactions involving M73, L76, and Y80 from both chains. Additional hydrophobic residues from helix $\alpha 1$ (L24 and Y28) and helix $\alpha 2$ (I39, I42, V43, L46, and L47) form contacts with the reciprocal monomer to facilitate robust dimer formation (Fig. 1d). The total buried surface area of the interface is $1383 \text{ \AA}^2$ (PISA server, http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html)¹¹.

A search for homologous protein structures using FATCAT^{12; 13}, VAST¹⁴ and DALI¹⁵ revealed no significant results, indicating that this is a novel protein fold. However, cryptic similarity to other helical bundle proteins may be obscured by the domain-swapped topology of IreB, the existence of which implies that monomeric IreB is not a stably folded protein. Alignment of the sequences of IreB homologs from a diverse collection of *Firmicutes* (Fig 2a) reveals a stretch of highly conserved residues that extends through helix $\alpha 2$ and the loop between $\alpha 2$ and $\alpha 3$. This loop is solvent-exposed for both monomers in the dimeric structure (Fig. 2b), and the high amino acid conservation suggests it is functionally critical. Consistent with this hypothesis, we previously identified mutations at D50 (D50A and D50G) that result in a loss of IreB function *in vivo*⁹.

IreB forms dimers *in vitro*

In light of the dimeric IreB structure observed by NMR and the conservation of interface residues (Fig 2a), we used additional biophysical approaches to test for dimerization of IreB in solution. Analysis of recombinant IreB by size-exclusion chromatography (Fig. 3a) revealed that IreB predominantly eluted with a retention time consistent with that of a dimer, and little monomeric IreB (10.5 kDa) was observed. Moreover, analytical ultracentrifugation (Fig. 3b) confirmed that IreB is a stable dimer at low micromolar concentrations in solution, thereby validating the dimeric structure observed by NMR.

IreB forms oligomers *in vivo*

To determine if IreB also exhibits dimer formation *in vivo* (i.e. in live *E. faecalis* cells), we used the protein-fragment complementation assay (PCA) based on reconstitution of murine dihydrofolate reductase (mDHFR) activity. Analogous to a traditional 2-hybrid assay, we previously showed¹⁶ that co-expression in *E. faecalis* of genetic fusions between interacting proteins and fragments of mDHFR (designated F_[1,2] and F_[3]) can result in reconstitution of mDHFR activity *in vivo*, dependent on interaction between the fused protein partners, thereby enabling growth in the presence of the bacterial DHFR inhibitor trimethoprim (TMP). Co-expression of IreB-F_[1,2] and IreB-F_[3] fusions in *E. faecalis* led to enhanced growth in the presence of TMP (Fig. 4a) that did not occur when either fusion was expressed alone or when IreB-F_[1,2] was co-expressed with a randomly chosen control fusion, EF1039-F_[3], indicating that IreB is capable of interacting with itself in live cells of *E. faecalis*.

To confirm the results of mDHFR PCA by a different approach, we performed coimmunoprecipitation studies on *E. faecalis* cell lysates. New *E. faecalis* strains were constructed that expressed only F_[3] fusions to either IreB or the randomly chosen control protein, EF1039 (i.e., no F_[1,2] fusions were present in these strains). Protein complexes were immunoprecipitated from whole-cell *E. faecalis* lysates using an anti-F_[3] antibody. We found that endogenous (unfused) native IreB coimmunoprecipitated with IreB-F_[3] (Fig. 4b). In contrast, native IreB was not recovered from a strain expressing the non-interacting control protein EF1039-F_[3], indicating the specificity of IreB-IreB coimmunoprecipitation (Fig. 4b). Thus, in combination with the *in vivo* PCA data, these results indicate that IreB oligomerizes in live cells of *E. faecalis*.

Oligomerization is important for IreB function

In the NMR structure of IreB, side chains from residues on helix $\alpha 4$ participate in numerous interactions at the dimer interface (Fig. 1c and 1d). To test if dimerization is functionally important for IreB *in vivo*, we introduced a series of mutations at sites along the length of helix $\alpha 4$ intended to disrupt the dimer interface, including at 2 well-conserved sites that participate in intersubunit contacts (M73 and Y80). The mutations introduced charged amino acids to promote electrostatic repulsion (arginine or glutamate), or alternatively introduced a bulky hydrophobic amino acid (tryptophan) in an attempt to sterically interfere with contacts at the dimerization interface. All mutations were initially constructed in the context of the IreB mDHFR fusions to assess their effect on *in vivo* IreB oligomerization. Growth in the presence of TMP for 2 mutants (Y79E and L81W) was not impaired in the mDHFR PCA (Fig 5), indicating that these substitutions can be accommodated without disruption of the IreB dimer. For Y79E this is perhaps not surprising, as Y79 is solvent-exposed in the structure, likely affording its side chain conformational flexibility that would enable it to tolerate introduction of charged residues. L81 is buried at the dimer interface, so presumably introduction of tryptophan at this site is not sufficiently bulky to disrupt dimerization. In contrast to these mutations, all other substitutions in helix $\alpha 4$ led to a substantial loss of the ability to grow in the presence of TMP, indicating that IreB oligomerization is compromised *in vivo*. Thus, introduction of bulky or charged residues at several sites along helix $\alpha 4$ within the dimer interface is sufficient to disrupt IreB dimerization *in vivo*.

To determine if dimerization is important for IreB function, we performed antimicrobial susceptibility assays. Although the molecular function of IreB is unknown, our previous work showed that IreB acts a negative regulator of cephalosporin resistance, because *E. faecalis* mutants lacking functional IreB exhibit dramatically elevated levels of resistance to broad-spectrum cephalosporins such as ceftriaxone compared to wild-type cells. This phenotype can be complemented by expression of wild-type IreB carrying a C-terminal Strep tag from a plasmid expression platform⁹. Therefore, we introduced the helix $\alpha 4$ mutations into *ireB* encoded in this plasmid and determined the level of ceftriaxone resistance of a *ireB* mutant host strain of *E. faecalis* carrying each of the resulting plasmids. As expected, expression of wild-type IreB in the *ireB* mutant reduced the level of ceftriaxone resistance to wild-type levels (Table 2). Similarly, expression of the oligomerization-competent IreB Y79E mutant reduced ceftriaxone resistance to near wild-type levels, indicating that IreB Y79E is largely functional. In contrast, none of the other

dimerization interface mutants could restore the level of ceftriaxone resistance in the *ireB* mutant (Table 2), indicating that these mutants are nonfunctional *in vivo*. Relative to most of the other mutants, the L81W mutant is unusual, in that it is nonfunctional but retains oligomerization *in vivo* (Fig 5). At this point it remains unclear why the IreB L81W mutant is nonfunctional. Residue L81 is highly conserved (Fig 2a), suggesting that L81 plays an important role in IreB function – independent of dimerization *per se* – that is perturbed by substitution with Trp. Regardless, overall these results establish a strong correlation between oligomerization and function of IreB *in vivo*, consistent with the hypothesis that dimerization of IreB *in vivo* is required (but not necessarily sufficient) for its function in the pathway controlling intrinsic cephalosporin resistance in *E. faecalis*. Immunoblot analysis using anti-IreB antiserum revealed that mutants for which IreB oligomerization is impaired also exhibit reduced steady-state IreB protein levels in *E. faecalis* cells (Figure 6), suggesting that dimerization is an important means of stabilizing IreB *in vivo*.

Modification of IreB impacts oligomerization of IreB *in vivo*

IreB can be phosphorylated at sites located on its flexible N-terminal end by the IreK kinase. Dephosphorylation of those sites occurs through the action of the cognate phosphatase, IreP; hence, mutants lacking IreP will accumulate IreB in its modified form. In previous work, we found that an IreB mutant unable to be phosphorylated at the known sites nevertheless exhibited an altered isoelectric point in an *E. faecalis* mutant lacking IreP⁹, indicating that IreB is also subject to an additional modification *in vivo* (potentially phosphorylation, but as-yet-unknown). To determine if post-translational modifications influence the ability of IreB to oligomerize *in vivo*, we used the mDHFR PCA to monitor IreB interactions in *E. faecalis* cells carrying a temperature-sensitive (ts) mutant of the IreP phosphatase (IreP F31T). This IreP(ts) mutant retains near wild-type activity at the permissive temperature of 30 °C (resulting in wild-type levels of cephalosporin resistance), but is inactivated at the nonpermissive temperature of 37 °C (yielding dramatically elevated levels of cephalosporin resistance characteristic of *ireP* mutants). Hence, growth of the *ireP(ts)* mutant at 37 °C will yield fully modified IreB. We co-expressed the IreB-F_[1,2] and IreB-F_[3] fusions in the *ireP(ts)* mutant and analyzed growth in the presence of TMP. At permissive temperature, IreB oligomerization occurred normally, as reflected by the wild-type growth in the presence of TMP (Fig. 7). In contrast, at nonpermissive temperature, growth of the *ireP(ts)* mutant was dramatically impaired, suggesting that fully modified IreB was unable to oligomerize efficiently.

Discussion

IreB is a small protein that acts, by an unknown mechanism, as a negative regulator of resistance towards cell-wall-active antimicrobials in *E. faecalis*. IreB can be phosphorylated by IreK, a transmembrane Ser/Thr kinase that is required for resistance to cell-wall active antimicrobials, and IreB mutants that cannot be phosphorylated constitutively repress resistance, rendering *E. faecalis* susceptible⁹. Although the molecular details remain unknown, it seems likely that IreB influences resistance to diverse cell-wall-active antimicrobials by modulating some aspect of cell wall integrity, synthesis, or homeostasis.

BLAST searches reveal that well-conserved homologs of IreB are widespread among bacteria, being encoded in the genomes of most members of the *Firmicutes* phylum (a representative set depicted in Fig 2a), but are rarely found outside the *Firmicutes*. The robust conservation among *Firmicutes* suggests that IreB homologs play an important role in the physiology of this group of bacteria, presumably serving a common function related to the integrity, synthesis, or homeostasis of the cell wall of these organisms. In *E. faecalis*, which is intrinsically resistant to many cell-wall-active antimicrobials, IreB action ultimately impacts antimicrobial resistance, a phenotype for which there exists a straightforward experimental readout. Consistent with our previous findings in *E. faecalis*, one phosphoproteomics study performed on *Streptococcus agalactiae* identified the *S. agalactiae* homolog of IreB as a phosphoprotein¹⁷, with phosphorylation occurring on the equivalent of residues T4 and T7 in *E. faecalis* IreB, although the function of the *S. agalactiae* protein was not established. Thr7 in particular is nearly universally conserved among IreB homologs. Thus it seems likely that reversible phosphorylation of IreB homologs to regulate their biological activity is also a widespread feature of this family of proteins.

To the best of our knowledge, a structure-function analysis on a member of the IreB family has not been described. We determined the NMR solution structure of IreB, revealing that IreB adopts a unique, highly alpha-helical fold encompassing most of the polypeptide. However, residues 1–17 (containing the known sites of phosphorylation, Thr4 and Thr7) were dynamically disordered on the picosecond to nanosecond timescale. Flexibility in this region likely facilitates entry into the catalytic cleft of the cognate IreK kinase for phosphorylation to occur. We imagine that phosphorylation could provide new opportunities for electrostatic interactions that could lead to a stable repositioning of the N-terminal segment, either on IreB itself or with another as-yet-unidentified binding partner. However, it remains unclear what the structural consequences (if any) of phosphorylation on IreB are. Our analysis of dimerization *in vivo* (Fig 7) suggests that phosphorylation might lead to loss of IreB homodimerization, but whether that behavior represents an inherent property of IreB itself or occurs due to, for example, phosphorylation-dependent changes in interactions with potential binding partners *in vivo* (e.g. a “partner-switching” mechanism) awaits further investigation.

A centrally located segment of IreB residues (E33 through R56) is highly conserved across IreB homologs from diverse *Firmicutes* (Fig 2a). The IreB structure revealed that a subset of these highly conserved residues comprise a solvent-exposed loop linking helices $\alpha 2$ and $\alpha 3$ (Fig. 2b). That the residues of this loop are nearly universally conserved implies that they are critical to IreB function, and their location on an exposed loop suggests that they could be involved in interactions with other cellular factors. Consistent with the likely functional significance of the loop, in our previous work we identified nonfunctional mutants of IreB that contained substitutions in this loop (D50A and D50G). Apart from this, however, the structure of IreB does not immediately suggest a likely biochemical function for this family of proteins.

The NMR structure of IreB revealed that it forms a dimer in solution and adopts a partially domain swapped conformation in which helix $\alpha 4$ from each monomer plays a critical role in intersubunit interactions (Fig. 1), contributing 776Å² (56%) to the total area of the interface.

Intermolecular contacts in helix 4 are highly conserved (Fig 2a), as L76 and Y80 are nearly invariant, and position 73 is always a methionine, isoleucine, or valine. Additional biophysical approaches confirmed that IreB is a stable dimer at low micromolar concentrations in solution (Fig. 3), thereby validating the dimeric structure observed by NMR. Moreover, dimerization of IreB was confirmed *in vivo*. The experimental approaches used to examine IreB dimerization *in vivo* cannot unequivocally distinguish dimers from higher-order oligomers, so it remains formally possible that IreB exists in higher-order oligomers *in vivo*, although based on the *in vitro* studies we suggest that dimers are the most likely form *in vivo* as well. Homodimer formation appears to be functionally important for the biological activity of IreB, because specific substitutions at the IreB dimer interface impaired both dimerization and IreB function and stability *in vivo*. Our observations with the IreB L81W mutant – namely, that it appears competent at dimerization, yet is non-functional *in vivo* – imply that dimerization of IreB is required for function but that dimerization, *per se*, may not be sufficient. In summary, these studies provide new insights into the structure and function of a widely conserved protein of unknown function that is an important regulator of antimicrobial resistance in *E. faecalis*.

Materials and Methods

Bacterial strains, growth media and chemicals

E. faecalis strains were grown in half-strength brain heart infusion broth for routine maintenance or Mueller Hinton broth (MHB) for experimental analyses. *E. coli* strains were grown in lysogeny broth (LB) for routine maintenance. Antibiotic concentrations were used as follows: chloramphenicol (Sigma) 10 µg/mL, kanamycin (GoldBio) 50 µg/mL, ampicillin (Sigma) 100 µg/mL, erythromycin (Goldbio) 10 µg/mL; trimethoprim (Sigma), 1 µg/mL. Bacterial strains and plasmids used in this study are listed in Table 3.

Construction and expression of IreB point mutants

For complementation and antimicrobial susceptibility studies, an expression vector based on pCI3340 with a constitutive promoter was used. PCR fragments encoding the P23 promoter of pDL278p23¹⁸ fused to *ireB* (or *ireB* point mutants) with a C-terminal strep tag were inserted between the EcoRI and HindIII restriction sites of pCI3340, as previously described⁹. For mDHFR-based PCA assays, fusions of *ireB* or non-specific control proteins to mDHFR fragments were co-expressed from constitutive promoters found in the compatible plasmids pBK200 and pJRG9, as previously described¹⁶. All plasmid clones were sequenced to verify the absence of errors.

Cloning, expression and purification of IreB

IreB and derivatives with amino acid substitutions were cloned into a modified pET28a (Novagen) vector that contains a Hexa-His tagged SMT3 (SUMO) fusion protein. BL21 *E. coli* containing the pREP4 plasmid (for *lacI* expression) were transformed with these pET28a-SMT3-IreB plasmids. For NMR analysis of wild-type IreB, one-liter cultures (LB media or U-¹⁵N or U-¹⁵N, ¹³C M9 minimal media) were grown at 37 °C to an optical density at 600 nm of 0.6–0.7. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for five hours after which cell pellets were collected by centrifuging

10 minutes at 5,000 g and stored at -80°C . Cells were resuspended in 10 mL of lysis/binding buffer (50 mM Na_2HPO_4 , pH 7.4, 300 mM NaCl, 10 mM imidazole) and lysed using a French Press (16,000 psi). The soluble lysate containing SMT3-IreB was loaded onto Ni-Agarose resin (Clontech), washed with Buffer A (50 mM Na_2HPO_4 , pH 7.4, 300 mM NaCl, 10 mM imidazole) and eluted with Buffer B (50 mM Na_2HPO_4 , pH 7.4 300 mM NaCl, 500 mM imidazole). Elutions were dialyzed against 4 L Na_2HPO_4 , pH 7.4, 150 mM NaCl and 400ug GB1-Ulp1 (Ubiquitin-like specific protease 1/SUMO-Protease 1) added in dialysis bag. After dialysis, the cleaved protein was loaded onto Ni-Agarose resin (GE Healthcare) and the flow through containing IreB was collected. IreB was then concentrated to 1 mM and buffer exchanged into 50 mM Na_2HPO_4 , pH 6.6, 350 mM NaCl. All purified wild-type and mutant IreB proteins were analyzed by circular dichroism for folding and by size exclusion chromatography for oligomeric state. All variants appeared to contain some helical structure based on CD spectra. SEC elution profiles suggested that IreB Y79E and L81W remained dimeric, while the other variants that exhibited a loss of dimerization *in vivo* yielded altered SEC profiles.

Structure of IreB by NMR

The sample used for NMR contained 1.0 mM [$U\text{-}^{13}\text{C},^{15}\text{N}$] IreB, 50 mM NaPi pH 6.6, 350 mM NaCl, and 0.02% azide in 95% H_2O and 5% $^2\text{H}_2\text{O}$. All NMR data were acquired at 45°C on a Bruker Avance 600 MHz spectrometer equipped with a triple-resonance CryoProbeTM and processed with NMR Pipe software¹⁹. The total acquisition time for all NMR spectra was 290 h. More than 90% of the backbone ^1H , ^{15}N and ^{13}C resonance assignments were obtained in an automated manner using the program Garant²⁰, with peaklists from 3D HNCOC, HNCACOC, HNCA, HN(CO)CA, HNCACB, and C(CO)NH spectra. Sidechain assignments were completed manually from 3D HC(CO)NH, HBHACONH, HCCH-TOCSY and $^{13}\text{C}(\text{aromatic})$ -edited NOESY-HSQC spectra analyzed with XEASY²¹. Heteronuclear ^{15}N - ^1H NOE values were determined from an interleaved pair of two-dimensional gradient sensitivity-enhanced correlation spectra of [$U\text{-}^{13}\text{C},^{15}\text{N}$] IreB acquired with and without a 3-sec proton saturation period²² and analyzed using CARA, freely available from <http://www.nmr.ch>. A total of 1017 unique non-trivial NOE distance constraints for each monomer were obtained from 3D ^{15}N -edited NOESY-HSQC, ^{13}C -edited NOESY-HSQC spectra, and $^{13}\text{C}(\text{aromatic})$ -edited NOESY-HSQC spectra ($\tau_{\text{mix}} = 80$ ms). An additional 138 intermolecular distance constraints were obtained from a 3D F1- ^{13}C -filtered/F3- ^{13}C -edited NOESY-HSQC spectrum²³ ($\tau_{\text{mix}} = 120$ ms). A total of 107 backbone ϕ and ψ dihedral angle constraints were generated from secondary shifts of the $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, $^{13}\text{C}'$, and ^{15}N nuclei shifts using the program TALOS+²⁴. Initial structures were generated in an automated manner using the NOEASSIGN module of the torsion angle dynamics program CYANA²⁵, along with manually assigned intermonomer restraints, as previously described²⁶. The structure was then further refined iteratively by the addition of manually-assigned NOEs and subsequent rounds of CYANA. Of the 100 CYANA structures calculated, the 20 conformers with the lowest target function were subjected to a molecular dynamics protocol in explicit solvent²⁷ using XPLOR-NIH²⁸.

Size exclusion chromatography

Size exclusion chromatography was performed using a Superdex 75 10/300 GL column (GE Healthcare) with a running buffer containing Na_2HPO_4 pH 7.4, 150 mM NaCl at flow rate of 0.4 mL min^{-1} . Mass of IreB oligomer estimated by comparison to standards (Albumin (66 kDa) ovalbumin (45 kDa), and RNase A (13.7 kDa). IreB was injected at 200 μM in 100 μL injection volume. Standards were run at 100 μg each in a 200 μL injection volume.

Sedimentation Equilibrium

A Beckman Optima XL-A analytical ultracentrifuge was used for sedimentation equilibrium experiments. Protein samples at 64, 32 and 16 μM in 20 mM Na_2HPO_4 with 350 mM NaCl at pH 6.6 were dialyzed against 2 liters of the same buffer overnight at room temperature. Double-sector charcoal-filled Epon 1.2-cm path length centerpieces were loaded with 100 μL of each sample in one sector and 110 μL of the furnished dialysate buffer in the reference sector. Concentration gradients were monitored at 277 nm with superimposable gradients recorded 2–3 or more hours apart taken as indicating equilibrium had been reached. Equilibrium data were collected at 12000, 15000, 18000, 21000, 28000 and 42000 rpm at 40 °C. Absorbance remaining near the meniscus after several hours at 50000 rpm was taken as a measure of non-sedimenting baseline optical density. The initial absorbances (277 nm) for the three samples were 0.782, 0.397 and 0.179 with baseline optical densities of 0.010, 0.027, and 0.000 respectively. The molecular weight and partial specific volume of IreB were calculated from the amino acid sequence to be 10,545 Da and 0.737 ml/g (at 40 °C)²⁹, respectively. The extinction coefficient calculated from the tryptophan and tyrosine content was $10,430 \text{ M}^{-1}\text{cm}^{-1}$ ³⁰. The density of the buffer was calculated using density increment approach^{31; 32} at 40 °C as 1.012 g/mL.

A program written for Igor Pro (Wavemetrics Inc., Lake Oswego, OR) by Darrell R. McCaslin was used for the analysis of the sedimentation equilibrium data. Log plots showed no curvature, indicative of a homogenous species. The data were initially fit globally to single and two species models, but the two species fit did not yield distinct species ($\text{Mw/Ms} = 2.15$ and 2.39). The reduced Mw from the global single-species analysis was 5489.7 ± 5.2 to yield $\text{Mw/Ms} = 2.05$. Residuals were evenly distributed between 0.03 and -0.03 AU, consistent with a high quality fit.

Antibiotic susceptibility determinations

The minimal inhibitory concentrations (MICs) for antibiotics were determined in aerobic liquid cultures using a microtiter-plate serial dilution method. Two-fold serial dilutions of ceftriaxone in MHB (plus chloramphenicol for plasmid maintenance) were prepared in 96-well plates. Bacteria from stationary-phase cultures were inoculated into each well to a density of $\sim 10^5 \text{ CFU/mL}$. Plates were incubated at 37°C for 24 hours and optical density at 600 nm was measured using a Molecular Devices Spectra Max MS plate reader. MIC was defined as the lowest concentration of drug that inhibited culture growth as measured by optical density at 600 nm after 24 hours.

Immunoblot of whole cell lysates

Stationary-phase cultures grown in MHB plus chloramphenicol for plasmid maintenance were diluted 1:100 in 20 mL fresh MHB (supplemented with chloramphenicol). The cultures were incubated at 37°C and harvested during exponential phase when optical density measured at 600 nm was approximately 0.2. To harvest, an equal volume of ethanol/acetone (1:1) mix was added to cultures to kill cells. The cell suspension was pelleted by centrifugation, washed twice with 1 mL water and stored at -20°C overnight. Thawed pellets were resuspended in 100 µL 20 mM Tris pH 7.5, 10 mM EDTA and treated with 5 mg/mL lysozyme for 20 minutes at 37°C. Samples were boiled for 10 minutes in Laemmli SDS sample buffer with 0.5 M DTT then subjected to SDS PAGE analysis and proteins were transferred to PVDF membrane using a semi-dry transfer apparatus. The membrane was blocked in 5% skim milk in Tris-base saline with 0.1% Tween 20 (TBST) and probed with anti-IreB antibody from rabbit serum (diluted 1:2000 in TBST) overnight at 4 °C. The next day the blot was washed in TBST and probed with HRP-conjugated goat anti-rabbit antibody (diluted 1:5000 in 5% skim milk in TBST). Detection was performed using PIERCE chemiluminescent kit according to the manufacturer's instructions.

Co-immunoprecipitation

A volume of 5 mL of stationary-phase culture grown was diluted 1:20 in 100 mL fresh medium and incubated at 37°C until optical density at 600 nm reached a value of 0.2. Cultures were pelleted, washed twice with 1 mL water and resuspended in 400 µL lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% Tween-20, 1 × Halt protease inhibitor cocktail (Thermo Scientific), 0.5 × Halt phosphatase inhibitor cocktail (Thermo Scientific). Lysis was performed by bead beating with 6 rounds of 30-second bursts and using 0.2-mM silica beads. The soluble lysates were recovered by centrifugation and an aliquot was removed to represent the input sample for later analysis by immunoblot. To the remaining lysates, 1.5 µL 1 µg/µL rabbit DHFR C-terminal antibody (Sigma) was added and the mixture was incubated at 4°C for 1 hour on a nutator. Protein A agarose beads (Invitrogen) that were previously blocked using BSA (1% BSA in lysis buffer incubated at 4°C for one hour on a nutator) were added to the mixture and incubation was continued for an additional hour to recover the DHFR C-terminal antibody and any protein bound to it. Protein A agarose beads were recovered using a micro Biospin column (Bio-rad), washed three times with 350 µL lysis buffer. Proteins bound to the Protein A agarose beads were recovered by boiling the beads in a volume of 25 µL 4X Laemmli SDS sample buffer with 0.5 M DTT. Input and immunoprecipitated samples were subjected to SDS-PAGE, transferred to PVDF membrane, blocked with 5% skim milk in TBST and then probed overnight at 4°C with the anti-DHFR C-terminal antibody to monitor recovery of IreB-F_[3] and rabbit anti-IreB antibody in serum to test for co-immunoprecipitation of the genomic encoded untagged IreB. The following day, the blots were probed with the secondary antibody HRP-conjugated goat anti-rabbit (diluted 1:5000 in 5% skim milk in TBST). Antibody reactive bands were detected using PIERCE chemiluminescent kit according to manufacturer instructions.

mDHFR PCA

We previously adapted the *in vivo* PCA based on murine dihydrofolate reductase (mDHFR) for use in *E. faecalis*¹⁶. Co-expression of mDHFR fusions in *E. faecalis* was achieved by introducing compatible expression plasmids (derivatives of pBK200 and pJRG9) that encoded C-terminal fusions of genes to be tested to the N-terminal ends of F_[1,2] or F_[3] mDHFR fragments, respectively. All fusions were to full-length *E. faecalis* proteins and included a (Gly₄Ser)₂ flexible linker between the protein of interest and the fused mDHFR fragment. Expression of the fusions was driven by the constitutive P23s promoter encoded in pBK200 and pJRG9. To test for reconstitution of mDHFR activity indicative of protein-protein association, a minimum of three independent transformants of *E. faecalis* co-expressing pairs of mDHFR fusions were grown on MH agar supplemented with Cm and Em (for plasmid maintenance) and trimethoprim at 1 µg/ml.

Construction of a temperature-sensitive *ireP* mutant of *E. faecalis*

We analyzed the IreP sequence for hydrophobic residues that may be disrupted to generate a temperature-sensitive phenotype. The method described by Varadarajan *et al.*^{33; 34} was used to identify F31T as a candidate substitution to provide temperature-sensitivity in IreP. The F31T substitution was introduced into the chromosomal allele of *ireP* in *E. faecalis* OG1RF using markerless allelic exchange, as previously described for *E. faecalis*³⁵. To assess the effects of the F31T substitution on IreP function, we analyzed cephalosporin resistance. Previous work showed that *E. faecalis* mutants entirely lacking IreP exhibit dramatically elevated resistance to cephalosporins compared to otherwise wild-type cells⁸. Comparison of cephalosporin resistance of wild-type OG1RF and the IreP F31T mutant (JSH1) on MH agar supplemented with increasing concentrations of ceftriaxone at 30 °C and 37 °C revealed that JSH1 exhibited near-wild-type levels of resistance at 30 °C, but highly elevated levels of resistance (comparable to the *ireP* mutant) at 37 °C (not shown), consistent with a loss of IreP function at elevated temperature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HSQC	heteronuclear single quantum coherence
NOE	nuclear Overhauser enhancement
NOESY	NOE spectroscopy
PCA	protein-fragment complementation assay
mDHFR	murine dihydrofolate reductase

TMP	trimethoprim
ts	temperature-sensitive
MHB	Mueller Hinton broth
LB	lysogeny broth
MIC	minimal inhibitory concentration

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Highlights

- IreB acts as a negative regulator of cephalosporin resistance in *E. faecalis* by an unknown mechanism
- Structural and biophysical studies reveal IreB is a dimer in solution with a novel fold
- IreB dimerizes *in vivo*
- Mutations that impair dimerization abolish regulation of cephalosporin resistance

Figure 1. NMR structure of IreB

(a) Ensemble of 20 final NMR conformers shown as Ca trace (PDB ID: 5US5). Residues 18–88 are shown for clarity. (b) Heteronuclear ^{15}N - ^1H NOE values measured at 14.1 T. Residues with overlapping signals were omitted. (c) IreB adopts a novel dimeric fold. Each IreB protomer contains 4 α -helices, with the α_4 helix partially domain-swapped. Side chains of selected interface residues are shown as sticks and labeled. (d) IreB dimer interface as viewed along the axis of twofold symmetry. Hydrophobic core residues that make intermolecular contacts involving the α_4 helix are labeled.

Figure 2. IreB contains a highly conserved helix and loop

(a) Sequence alignment of homologs of IreB from a diverse collection of *Firmicutes* identifies a region (highlighted in orange) of highly conserved sequence encompassing the $\alpha 2$ helix and adjoining loop residues. The locations of helical secondary structural elements are depicted below the sequences. Conserved dimer interface residues are highlighted in blue. Sequences were: *Enterococcus faecalis* V583 (gi 29375777); *Streptococcus mutans* UA159 (gi 24380414); *Lactococcus lactis* subsp. *lactis* Il1403 (gi 15672124); *Lactobacillus paracasei* ATCC 334 (gi 116494330); *Bacillus subtilis* 168 (gi 50812272); *Staphylococcus aureus* NCTC8325 (gi 88195426); *Clostridium difficile* Y384 (gi 531633012); *Listeria monocytogenes* EGD-e (gi 16803543); *Leuconostoc mesenteroides* (gi 490267964); *Pediococcus claussenii* (gi 503981218); and *Halobacillus halophilus* 504456746). Alignment was generated by CLUSTAL W (1.83). (b) Conserved $\alpha 2$ helix and $\alpha 2$ - $\alpha 3$ loop residues are located in a solvent-accessible region of the the IreB structure (orange) away from the dimer interface with the opposing IreB monomer (blue surface).

Figure 3. IreB forms a dimer *in vitro*

(a) Size exclusion chromatography of purified recombinant IreB was performed using a Superdex 75 10/300 GL column. The retention time for IreB (black) was compared to protein standards (blue). Monomeric IreB is 10.5 kDa. (b) Absence of curvature in log plots of equilibrium analytical ultracentrifugation data (open circles; after subtraction of baseline optical density) for IreB at initial concentrations of 16 (blue), 32 (red) and 64 (green) μ M showed that the protein sediments as a non-interacting single species. Global nonlinear fitting (black lines) to a single species model yielded a M_w/M_s ratio of 2.05, consistent with a stable IreB dimer.

Figure 4. IreB oligomerizes *in vivo*

(a) *E. faecalis* strains co-expressing the indicated fusions were subjected to 10-fold serial dilutions and inoculated on MH agar supplemented with Cm and Em (for plasmid selection) in the presence or absence of trimethoprim. Co-expression of IreB fusion proteins reconstituted mDHFR activity and led to enhanced growth in the presence of trimethoprim. EF1039 is a randomly chosen *E. faecalis* control protein that demonstrates the specificity of the IreB-IreB *in vivo* association. The host strain was an *E. faecalis* *ireB* mutant (CK164).

(b) IreB oligomerization in whole-cell lysates of *E. faecalis* was assessed by coimmunoprecipitation using anti-F_[3] antiserum. IreB or a randomly chosen *E. faecalis* control protein (EF1039) fused to mDHFR F_[3] (as indicated, top) was expressed in otherwise wild-type *E. faecalis* cells. Lysates and immunoprecipitates were subjected to immunoblot analysis with anti-IreB antiserum, revealing that native (untagged) IreB

specifically coprecipitates with IreB-F_[3]. The host strain was *E. faecalis* OG1RF. pIreB-DHFR F_[3] is pLMS118; pEF1039-DHFR F_[3] is pHS14.

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Figure 5. Mutations at the dimer interface impair oligomerization *in vivo*

(a) IreB dimer interface. Mutated residues are labeled. (b) *E. faecalis* strains co-expressing the indicated fusions were subjected to 10-fold serial dilutions and inoculated on MH agar supplemented with Cm and Em (for plasmid selection) in the presence or absence of trimethoprim. Co-expression of interaction-competent IreB fusion proteins reconstituted mDHFR activity and led to enhanced growth in the presence of trimethoprim, while interaction-defective mutants do not support growth. The host strain was an *E. faecalis* *ireB* mutant (CK164).

Figure 6. Mutations at the dimer interface influence IreB levels

Whole-cell lysates of exponentially growing cultures were probed with anti-IreB antibody or anti-EF0233 as a loading control. Biological replicates are shown (1 and 2) for each strain. Except for the first lane in each panel, samples were derived from the *ireB* mutant (CK164) carrying an *ireB* expression plasmid. Plasmids: vector was pCI3340, wild-type was pCJK187, M73R was pCLH172, M73W was pCLH173, Y79E was pCLH171, Y80E was pCLH174, L81R was pCLH175, L81W was pCLH176.

Figure 7. Modification of IreB impacts oligomerization *in vivo*

E. faecalis strains co-expressing the indicated fusions were subjected to 10-fold serial dilutions and inoculated on MH agar supplemented with Cm and Em (for plasmid selection) in the presence or absence of trimethoprim. Plates were incubated at the permissive temperature (30 °C) or non-permissive temperature (37 °C) to inactivate IreP phosphatase activity and drive IreB modification. Host strains were *E. faecalis* OG1RF (WT) or an isogenic mutant carrying the *ireP(ts)* mutation (JSH1).

Table 1

Statistics for the 20 IreB conformers

Experimental constraints				
Distance constraints for each monomer				
Intermolecular				138
Lon				224
Medium [$1 < (i-j) \leq 5$]				252
Sequential [$(i-j)=1$]				267
Intraresidue [$i=j$]				274
Total				1155
Dihedral angle constraints (ϕ and ψ)				107
Number of restrains per residue				14.2
Number of long-range restrains per residue				4.1
Average atomic R.M.S.D. to the mean structure (Å)				
Monomer	A	B	<A> vs 	A + B
Residues	18–86	18–86	18–86, 18–86	18–86, 18–86
Backbone (C $^{\alpha}$, C $^{\prime}$, N)	0.49 $\pm$ 0.09	0.53 $\pm$ 0.09	0.15	0.66 $\pm$ 0.14
Heavy atoms	1.06 $\pm$ 0.09	1.11 $\pm$ 0.10	0.24	1.17 $\pm$ 0.12
Deviations from idealized covalent geometry				
Bond lengths	RMSD (Å)		0.012	
Torsion angle violations	RMSD (°)		1.2	
Constraint violations				
NOE distance	Number > 0.5 Å ^a		0 $\pm$ 0	
NOE distance	RMSD (Å)		0.013 $\pm$ 0.001	
Torsion-angle violations	Number > 5 ° ^b		0.0 $\pm$ 0	
Torsion-angle violations	RMSD (°)		0.522 $\pm$ 0.088	
Global quality scores (raw/Z score) ^c				
Verify3D			0.41/–0.80	
Prosall			0.72/0.29	
PROCHECK (ϕ – ψ) ^d			0.28/1.42	
PROCHECK (all) ^d			0.15/0.89	
MolProbity clash score			19.45/–1.81	
Ramachandran statistics (% of all residues) ^e				
Most favored			94.4	
Additionally allowed			5	
Generously allowed			0.5	
Disallowed			0.1	

^aThe largest NOE violation in the ensemble of structures was $0.35 \text{ \AA}$.

^bThe largest torsion-angle violation in the ensemble of structures was 4.4°.

^cCalculated using PSVS version 1.5

^dBased on ordered residues 18–89 and 218–289.

^fRamachandran statistics calculated using PROCHECK for residues 18–89 and 218–289.

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Table 2Mutations at the dimer interface impair IreB function *in vivo*.

<i>E. faecalis</i> strain and plasmid			
Strain	Plasmid	Allele of <i>ireB</i>	MIC ^a (µg/ml)
<i>ireB</i>	pCI3340	none	256
<i>ireB</i>	pCJK187	wild-type	16
<i>ireB</i>	pCLH172	M73R	256
<i>ireB</i>	pCLH173	M73W	128
<i>ireB</i>	pCLH171	Y79E	32
<i>ireB</i>	pCLH174	Y80E	256
<i>ireB</i>	pCLH175	L81R	256
<i>ireB</i>	pCLH176	L81W	256

^aMedian minimal inhibitory concentrations (MICs) determined from 3 independent biological replicates after 24 h at 37 °C.

Table 3

Strains and plasmids used in this study

Strains or plasmids	Relevant description or genotype	Source or reference
Strains		
<i>E. coli</i>		
DH5α	Routine cloning host	Lab stock
BL21 (DE3)	Protein over-expression host	Lab stock
<i>E. faecalis</i>		
OG1RF	Wild-type reference strain (MLST 1)	36
CK164	OG1RF <i>ireB2</i>	9
JSH1	OG1RF <i>ireP F31T</i> (temperature-sensitive)	This work
Plasmids		
pCI3340	<i>E. coli</i> / <i>E. faecalis</i> shuttle vector (Cm ^R)	37
pBK200	<i>E. faecalis</i> expression vector, constitutive p23 promoter (Em ^R)	16
pJRG9	<i>E. faecalis</i> expression vector, constitutive p23 promoter (Cm ^R)	16
pCJK187	pCI3340:: p23- <i>ireB-strep</i>	9
pCLH171	pCI3340:: p23- <i>ireB Y79E-strep</i>	This work
pCLH172	pCI3340:: p23- <i>ireB M73R-strep</i>	This work
pCLH173	pCI3340:: p23- <i>ireB M73W-strep</i>	This work
pCLH174	pCI3340:: p23- <i>ireB Y80E-strep</i>	This work
pCLH175	pCI3340:: p23- <i>ireB L81R-strep</i>	This work
pCLH176	pCI3340:: p23- <i>ireB L81W-strep</i>	This work
pCLH178	IreB M73W-F _[1,2] fusion in pBK200	This work
pCLH179	IreB Y79E-F _[1,2] fusion in pBK200	This work
pCLH184	IreB Y80E-F _[1,2] fusion in pBK200	This work
pCLH185	IreB L81R-F _[1,2] fusion in pBK200	This work
pCLH186	IreB L81W-F _[1,2] fusion in pBK200	This work
pCLH187	IreB M73R-F _[3] fusion in pJRG9	This work
pCLH188	IreB M73W-F _[3] fusion in pJRG9	This work
pCLH189	IreB Y79E-F _[3] fusion in pJRG9	This work
pCLH190	IreB Y80E-F _[3] fusion in pJRG9	This work
pCLH191	IreB L81R-F _[3] fusion in pJRG9	This work
pCLH192	IreB L81W-F _[3] fusion in pJRG9	This work
pCLH204	IreB M73R-F _[1,2] fusion in pBK200	This work
pHS14	EF1039-F _[3] fusion in pJRG9	16
pLMS118	IreB-F _[1,2] fusion in pBK200	This work
pLMS180	IreB-F _[3] fusion in pJRG9	This work