Genetic analysis of signal integration by the *Sinorhizobium meliloti* sensor kinase FeuQ

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Two-component signalling systems allow bacteria to recognize and respond to diverse environmental stimuli. Auxiliary proteins can provide an additional layer of control to these systems. The *Sinorhizobium meliloti* FeuPQ two-component system is required for symbiotic development and is negatively regulated by the auxiliary small periplasmic protein FeuN. This study explores the mechanistic basis of this regulation. We provide evidence that FeuN directly interacts with the sensor kinase FeuQ. Isolation and characterization of an extensive set of FeuN-insensitive and FeuN-mimicking variants of FeuQ reveal specific FeuQ residues (periplasmic and intracellular) that control the transmission of FeuN-specific signalling information. Similar analysis of the FeuN protein highlights short patches of compatibly charged residues on each protein that probably engage one another, giving rise to the downstream effects on target gene expression. The accumulated evidence suggests that the periplasmic interaction between FeuN and FeuQ introduces an intracellular conformational change in FeuQ, resulting in an increase in its ability to remove phosphate from its cognate response regulator FeuP. These observations underline the complex manner in which membrane-spanning sensor kinases interface with the extracytoplasmic environment and convert that information to changes in intracellular processes.

**INTRODUCTION**

Bacteria use two-component systems (TCSs) to recognize and respond to a wide variety of external stimuli (Gao & Stock, 2009). Typically, TCSs consist of a membrane-bound sensor histidine kinase (SK) and its cognate response regulator (RR). For active kinase activity, the SK binds ATP and phosphorylates itself on a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain of the cognate RR (Casino *et al.*, 2010; Hoch, 2000; Russo & Silhavy, 1993). The RR often acts as a transcriptional regulator of genes functionally related to the detected stimulus (Casino *et al.*, 2010; Hoch, 2000; Russo & Silhavy, 1993). The combination of autophosphorylation of the SK and phosphotransfer to the RR will be referred to hereafter as kinase activity. In many cases, the SK can also serve as a phosphatase, facilitating removal of phosphate from the cognate RR (Ninfa & Magasanik, 1986). Depending on the concentration of the external stimulus, an SK can be toggled between activation (kinase-dominant) and inhibition (phosphatase-dominant) states.

The manner in which stimuli control the kinase and phosphatase activities of the SK proteins is not well understood and appears to vary in the broad family of SK proteins. However, biochemical and structural studies of numerous SKs have identified domains that participate in the kinase and phosphatase activities and their control (shown in Fig. 1a). The non-conserved nature of these sensor domains is probably due to the variation in stimuli sensed by different SKs (Pioszak & Ninfa, 2003b). On the cytoplasmic side of the membrane, the SK may have a membrane-proximal HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and some phosphatases) domain that connects transmembrane sensory inputs to output responses (Parkinson, 2010). Structural changes in the HAMP domain have been shown to control the signalling state of SKs (Ferris *et al.*, 2012; Parkinson, 2010). The cytoplasmic signalling module of an SK comprises two additional domains: a dimerization/histidine phosphorylation (DHp) domain and a C-terminal catalytic/ATP-binding (CA) domain (Ferris *et al.*, 2012). Mounting evidence suggests that complex interactions between cytoplasmic HAMP, DHp and CA domains control the activation or inhibition states of an SK. However, for many SKs, how the periplasmic sensing domain is linked

**Abbreviations:** Ap, ampicillin; Ara, L-arabinose; BACTH, bacterial adenylate cyclase two-hybrid; CA, C-terminal catalytic/ATP binding; DHp, dimerization/histidine phosphorylation; HAMP, histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins and some phosphatases; Km, kanamycin; RR, response regulator; SK, sensor histidine kinase; Tc, tetracycline; TCS, two-component system; TTT, targeted tri-threonine.

Three supplementary tables and three supplementary figures are available with the online Supplementary Material.
across the membrane to the cytoplasmic control of activation or inhibition states is poorly understood.

An increasing number of small (<200 aa) auxiliary regulator proteins have been discovered which allow for more precise regulation of TCSs. These auxiliary proteins have been identified in all cellular compartments, and their mechanisms of action vary from one system to another (Buelow & Raivio, 2010). Auxiliary proteins have been shown to link multiple TCSs to one another, to interrupt phosphorylation, phosphotransfer or phosphatase activities (Buelow & Raivio, 2010). Auxiliary proteins have been discovered which allow for more precise regulation of TCSs. These auxiliary proteins have.

The FeuQ-PQ TCS is required for functional symbiotic interaction between Sinorhizobium meliloti and compatible legumes (Griffitts et al., 2008). The FeuQ-PQ regulon consists of at least 16 target genes, including the symbiotically important gene nvdA. In a recent study a potential FeuP binding site motif was identified along with additional putative gene targets (Schlüter et al., 2013). The FeuQ-PQ regulon is stimulated in certain genetic backgrounds with defects relating to cell polarity and division control (Fields et al., 2012; Pini et al., 2013). Most recently, the FeuQ-PQ regulon has been found to be induced by several cationic antimicrobial peptides, including a nodule-specific cysteine-rich (NCR) peptide that is thought to stimulate rhizobial differentiation during symbiosis with host plants (Penterman et al., 2014).

We previously reported that the small periplasmic protein FeuN is an auxiliary regulator of the FeuPQ TCS in S. meliloti (Carlyon et al., 2010). FeuN negatively regulates FeuPQ signalling by acting upon the SK FeuQ, but the precise mechanism of this interaction is not known. Loss of FeuN is lethal to S. meliloti when FeuP and FeuQ are left intact, presumably due to overexpression of downstream genes (Carlyon et al., 2010). This presents a challenge for conducting mechanistic studies in S. meliloti. Fortunately, the FeuPQN system can be reconstituted in E. coli, which does not have an orthologous native signalling system. In both organisms, FeuN acts from its periplasmic location to modulate the cytoplasmic biochemistry occurring between FeuQ and FeuP, with output easily monitored using a P.screenlacZ reporter fusion (Carlyon et al., 2010).

In the work reported here, we take advantage of the E. coli heterologous system to conduct a detailed genetic dissection of the FeuN/FeuQ regulatory interaction. Our data point to a specific periplasmic FeuN/FeuQ binding interface that modulates the state of FeuP in the cytoplasm.

### METHODS

**Bacterial culture techniques.** Strains used in this study are detailed in Table 1. E. coli strains were cultured in Luria–Bertani (LB) medium supplemented as appropriate with ampicillin (Ap, 100 μg ml⁻¹), kanamycin (Km, 30 μg ml⁻¹), tetracycline (Tc, 10 μg ml⁻¹), L-arabinose (Ara, 0.3%) and X-Gal (50 μg ml⁻¹). Inducing conditions for the P, promoter were never used in this study.

**Plasmid construction.** Plasmids used in this study are listed in Table 1, and in Tables S2 and S3 (available in the online Supplementary Material). Detailed descriptions outlining the construction of these plasmids can be found in the Supplementary Methods section and in Tables S2 and S3. All PCR-generated inserts were verified by Sanger sequencing. All primers used for plasmid construction are listed in Tables S1, S2 and S3.

**Bacterial adenylate cyclase two-hybrid (BACTH) analysis.** Two-hybrid screens were performed using the pKT25, pUT18 and pUT18C
plasmids from the BACTH kit and the BTH101 indicator strain (EuroMedex). In this split-enzyme system, when the T18 and T25 fragments of *Bordetella pertussis* adenylate cyclase are in contact, they create an active enzyme that produces cAMP, and this can be assayed by monitoring  β-galactosidase activity (Karimova et al., 1998). Control plasmids encoding a leucine zipper (GCN4 from *Saccharomyces cerevisiae*) fused to the T18 and T25 fragments are included in the BACTH kit. Strains were suspended in water and diluted 100-fold, and 1 μl was plated on LB-Ap/Km/X-Gal. Colonies were grown at 30 °C for 48 h.

β-Galactosidase assays. For measuring *P_{nadA}–lacZ* expression in *E. coli*, bacterial culture and β-galactosidase assays were carried out at 30 °C. Stationary phase cultures were diluted 100-fold and grown for 6 h, at which point assays were carried out according to Miller (1972). Activity was also observed qualitatively by growing on plates containing X-Gal with or without Ara.

Random mutagenesis. Random mutagenesis PCR was performed using *Taq* and VentR polymerases (NEB) on eight separate templates.
in parallel to improve diversity of mutations. The amplification products were digested and ligated downstream of feuP in the pJG376 vector. The ligation mixture was transformed into E. coli DH5α/pG286 followed by plating on LB-Tc/Ap/Km/X-Gal. White colonies (indicating FeuN-mimicking phenotype) were restreaked and restested quantitatively with a β-galactosidase test as shown in Fig. S1.

**Targeted tri-threonine (TTT) scan of feuQ and feuN.** feuQ TTT substitutions were created using overlap-extension PCR across the region of feuQ corresponding to periplasmic residues D43–R168. feuN TTT substitutions were created using overlap-extension PCR across the region of feuN corresponding to the mature peptide (residues S36–K80). Detailed descriptions of the feuQ and feuN substitutions are provided in Table S3.

**RESULTS**

**Bacterial two-hybrid analysis indicates a physical interaction between FeuN and FeuQ**

To determine whether FeuN and FeuQ interact directly, the mature sequence of the periplasmic protein FeuN (Carlyon et al., 2010) and the periplasmic domain of FeuQ (R34–R171) were tested in a BACTH assay. We fused FeuN to the T25 fragment of B. pertussis adenylate cyclase, and the periplasmic domain of FeuQ to the T18 fragment. The BACTH kit includes leucine zipper control fusions to both the T25 and the T18 fragments (T18-zip and T25-zip). These T18 and T25 fusions were expressed in various combinations in the E. coli indicator strain BTH101 and plated on X-Gal (Fig. 1b). The data indicate a direct interaction between the FeuN–T25 fusion and both the FeuQ–T18 and the T18–FeuQ fusions. No interaction was detected in any of the negative control combinations. Our attempts to detect a physical interaction between FeuN and membrane-bound FeuQ using a spheroplast pull-down experiment were unsuccessful, suggesting that the interaction may be transient (data not shown).

**Does the FeuN/Q interaction cause an increase in FeuQ phosphatase activity?**

We previously showed that in the presence of FeuN, FeuQ has an observable negative regulatory activity (Carlyon et al., 2010). This negative regulation is presumably due to phosphatase activity. If FeuQ has both kinase and phosphatase activities, we hypothesized that selective elimination of kinase activity by mutation would create dominant-negative variants due to unchecked phosphatase activity. This is consistent with observations in other bifunctional sensor kinases (Atkinson & Ninfa, 1993; Hsing & Silhavy, 1997; Tanaka et al., 1998). To test this hypothesis, we created two FeuQ variants that were predicted to eliminate kinase activity while potentially leaving phosphatase activity intact. FeuQ (H255A) lacks the critical conserved His residue that is the site of autophosphorylation. An identical mutation in the well-characterized SK EnvZ was shown to eliminate kinase but not phosphatase activity (Hsing & Silhavy, 1997). Another variant, FeuQ (G398A), changes a highly conserved Gly residue previously shown to be critical for ATP binding and autophosphorylation (Tanaka et al., 1998). In the experiment shown in Fig. 2(a), the H255A and G398A variants were each co-expressed with FeuP. As predicted, both FeuQ variants displayed constitutively inhibiting phenotypes. This signalling inhibition is...
presumably due to intact phosphatase activity dominating the now disabled kinase activity.

To determine if FeuN can increase the signal inhibition of our kinase-deficient FeuQ allele, the FeuQ (H255A) variant was measured in the presence and absence of FeuN. For the experiment shown in Fig. 2(b), feuQ alleles were expressed from an uninduced P_{BAD} promoter, thus ensuring a very low FeuQ concentration in the cell. This is required because FeuQ (H255A) expressed from the uninduced P_{lac} system tends to bring reporter gene activity down to very low levels, obscuring any additional effects that may be brought about by FeuN. From the uninduced P_{lac} promoter, which transcribes at higher levels, we expressed a bicistronic feuP–feuN cassette. Where appropriate, the non-functional feuN(G61R) allele (Carlyon et al., 2010) is used as a negative control. Under these conditions, FeuN is indeed able to enhance the already inhibitory effect of FeuQ (H255A). This is consistent with the notion that FeuN directly enhances FeuQ phosphatase activity.

**FeuQ mutations lead to both FeuN-insensitive and FeuN-mimicking phenotypes**

While it seems clear that FeuN causes some shift in FeuQ that stimulates phosphatase activity, the mechanism of this shift is unclear. We performed random mutagenesis of feuQ searching for ‘FeuN-insensitive’ (constitutively activating) and ‘FeuN-mimicking’ (constitutively inhibiting) alleles that might reveal domains of the FeuQ protein that are important for potential FeuN interaction and toggling between kinase and phosphatase activities. We expected to find mutations leading to each phenotype in the HAMP, DHp and CA domains, as these regions are known to be involved in SK signal integration (Atkinson & Ninfa, 1993; Hsing et al., 1998; Willett & Kirby, 2012). We suspected that mutations might also correspond to the FeuN periplasmic domain, where FeuQ and FeuN are most likely to directly interact.

To identify FeuN-mimicking alleles of feuQ, we created a mutant library of feuQ and expressed library members in a strain with wild-type feuP and the P_{nadA}–lacZ reporter. We then screened for white colonies on X-Gal plates. Conveniently, null alleles of FeuQ appear light blue in this system due to low-level, non-specific phosphorylation of FeuP, while white colonies correspond only to FeuQ variants with functional negative regulatory activity (the FeuN-mimicking phenotype). As expected, we found FeuN-mimicking alleles clustering to the HAMP, DHp and CA domains (Fig. 3). Interestingly, we also found mutations clustering to the transmembrane segments and the N- and C-terminal ends of the periplasmic domain. These mutations highlight residues that potentially mediate the transfer of FeuN binding information into the cytoplasm. The screen for random FeuN-insensitive feuQ mutants was described previously (Carlyon et al., 2010). Briefly, a feuQ mutant library was expressed with wild-type feuP, wild-type feuN and the P_{nadA}–lacZ reporter and screened on X-Gal plates.

Under these conditions, most colonies are white due to FeuN/Q inhibition of the reporter gene, while dark blue colonies identify FeuN-insensitive FeuQ mutants. The mutations identified in this screen also clustered to amino acids in or flanking the transmembrane domain, in addition to a single mutation in the HAMP domain and a single mutation in the CA domain (see Fig. 3).

Given that FeuN is a periplasmic protein, one might expect that FeuN-insensitive behaviour might arise from amino acid changes to the FeuQ periplasmic domain. However, our random mutagenesis screen did not identify such a class. We performed a TTT amino acid substitution scan of the FeuQ periplasmic domain to look for FeuN-insensitive mutants. For TTT scanning, consecutive triplets of residues were changed to TTT. TTT substitution variants were made in FeuQ residues 43–168, covering most of the predicted periplasmic domain. The FeuQ variants were co-expressed with FeuP in our E. coli system, allowing for arabinose-inducible control of FeuN. Each allele was tested both on X-Gal plates and by quantitative β-galactosidase assays for signalling in the presence or absence of arabinose. The TTT scan of the FeuQ periplasmic domain revealed substitutions resulting in all four possible FeuQ phenotypes [see Fig. 4(a) and quantified data in Fig. S1]: wild-type (34/42), FeuN-mimicking (4/42), FeuN-insensitive (3/42) and non-functional (1/42). Wild-type alleles show normal reporter gene activation and FeuN responsiveness. FeuN-mimicking alleles mimic FeuN/Q inhibition even in the absence of FeuN. FeuN-insensitive mutants show reporter gene activation that is only slightly decreased when FeuN is co-expressed (none of the alleles was 100 % FeuN-insensitive). Of particular interest were two adjacent TTT mutants at FeuQ residues 139ETE141 and 142VVL144, both of which gave rise to an FeuN-insensitive phenotype. This TTT scanning approach reveals the critical role of the FeuQ periplasmic domain in governing responses to FeuN expression.

**TTT scan of mature FeuN peptide shows regions critical for FeuN function**

We performed a targeted TTT scan to look for critical FeuN regions potentially involved in its interaction with FeuQ (Fig. 4c). TTT substitutions were introduced into the mature FeuN peptide from residues 36 to 80 (a signal peptide cleavage site is predicted between residues 32 and 33). These FeuN variants were cloned into our E. coli system under control of the arabinose promoter. Strains were assayed with and without arabinose. The FeuN (TTT) phenotypes were characterized by the per cent loss of FeuN activity compared with the wild-type. The majority of TTT substitutions showed less than 5 % loss of function. Four alleles showed a 35–60 % loss of function, while only three alleles showed loss of function between 68 and 80 % (Figs 4c and S2). These three substitutions highlight areas potentially involved in interaction with FeuQ. None of the FeuN (TTT) variants was 100 % inactive.
Conserved compatibly charged residues within FeuQ and FeuN are required for FeuN/Q interaction

The study of amino acid residues critical for function in both FeuN and the periplasmic region of FeuQ highlighted potential regions of direct interaction between the two proteins. Our attention was drawn to a region of the FeuQ periplasmic domain where a negatively charged patch (139ETE141) leads to FeuN insensitivity when mutated to TTT (see Fig. 4a). Interestingly, the FeuN TTT scan revealed a functionally critical positively charged (73RPKK76) region near the C-terminal end of the protein (see Fig. 4c). In

![Diagram of FeuQ and FeuN interactions]

Fig. 3. Amino acid changes in FeuQ that cause FeuN-insensitive or FeuN-mimicking phenotypes. Left: schematic map of mutations as they correspond to conserved SK domains. Right: predicted structure of FeuQ cytoplasmic domains generated by the SWISS-MODEL Workspace (Arnold et al., 2006; Kiefer et al., 2009). Selected mutations are mapped to the structure with side chains displayed as points of reference.

Fig. 4. Map of TTT scan phenotypes of FeuQ (periplasmic domain) and FeuN. (a) Map of phenotypes caused by TTT substitutions across the periplasmic region of FeuQ (D43–R168). Detailed descriptions of these substitutions are given in Table S3. Each box represents three amino acid residues. Where the TTT substitution caused a change in signalling phenotype, the wild-type sequence is annotated. Charged residues important for subsequent studies are shown in bold. Quantitative β-galactosidase activity of each TTT mutant is shown in Fig. S1. (b) Representative photographs of FeuN-mimicking, wild-type and FeuN-insensitive phenotypes when tested on X-Gal plates. (c) Map of phenotypes caused by TTT substitutions across FeuN (S36–K80, corresponding to the mature peptide). Detailed descriptions of these substitutions are given in Table S3. Phenotypes are reported as the per cent loss of FeuN function compared with the unaltered protein. β-Galactosidase activity of each TTT mutant is given in Fig. S2.
alignments with homologous proteins from other alpha-proteobacteria, the FeuQ ETE and FeuN RPKK patches show strong charge conservation (Fig. 5a). We hypothesized that a direct FeuN/FeuQ interaction may involve these conserved oppositely charged sequence motifs. According to this model, one would predict that charge-reverse mutations in these regions would enhance the disruption of the interaction beyond what was seen in the charge-neutralizing TTT substitutions originally used to discover them. To test this prediction in FeuQ, the single charge-reversal mutants (TTT→KTT) and (TTT→TTK), as well as a double charge-reversed (TTT→KTK) variant were tested in the presence or absence of FeuN (Fig. 5b). The KTT and TTK variants showed a slight enhancement in FeuN insensitivity compared with the TTT mutation, consistent with our expectation. Surprisingly, the KTK variant with its double positive charge became strongly FeuN-mimicking. These charge-reversal phenotypes associated with the FeuQ 139ETE141 region underline its importance in periplasmic signal recognition and the transfer of that information into the cytoplasm.

We performed the reciprocal experiment on FeuN, creating three single charge-reverse variants at the critical RPKK sequence: FeuN (RPKK→EPKK), (RPKK→RPEK) and (RPKK→RPKE) (Fig. 5c). The RPEK variant was minimally affected, but the EPKK and RPKE variants were completely non-functional. This is consistent with our prediction that charge-reverse mutants would display greater loss of function than what was observed in our original TTT mutants. To confirm the stability and correct localization of the non-functional EPKK and RPKE variants, PhoA fusions to the C terminus of these variants were

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**Fig. 5.** Conserved, compatibly charged residues in the periplasmic region of FeuQ and in FeuN are involved in target gene regulation. (a) Sequence alignments of the relevant regions of FeuQ and FeuN. Conserved charged regions implicated in the TTT mutant screens are underlined. Alphaproteobacterial strains used for this comparison are *Brucella abortus* 2308, *Bartonella henselae* Houston 1, *Mesorhizobium loti* BNC1, *Rhizobium etli* CFN42 and *Agrobacterium tumefaciens* C58. (b) Analysis of the FeuQ ETE motif. $P_{\text{lac}}$ expression plasmids used were: pJG377 (wild-type *feuP–feuQ*), pJG376 (*feuP*-only), the original *feuP–feuQ*(ETE→TTT) clone, pJG685 (*feuP–feuQ*(ETE→KTT), pJG686 (*feuP–feuQ*(ETE→TTK)) and pJG687 (*feuP–feuQ*(ETE→KTK)). The $P_{\text{BAD–feuN}}$ plasmid pJG355 is included in all strains to monitor responsiveness to FeuN expression. For (b) and (c), only basal-level expression from $P_{\text{lac}}$ was allowed. (c) Analysis of the FeuN RPKK motif. The $P_{\text{lac}}$ plasmid pJG377 (*feuP–feuQ*) is included in all strains. The $P_{\text{BAD–feuN}}$ plasmids include pJG355 (*feuN*), pJG692 (*feuN(RPKK→EPKK)*), pJG693 (*feuN(RPKK→RPKE)* and pJG694 (*feuN(RPKK→RPKE)*).
tested for alkaline phosphatase activity. Both displayed alkaline phosphatase activity similar to that of the PhoA fusion to wild-type FeuN (Fig. S3). From these data it appears that the FeuN/FeuQ interaction is strongly influenced by these compatibly charged FeuQ ETE and FeuN RPKK sequences, with the internal lysine residue in the FeuN RPKK sequence playing a minor role.

We selected some of our charge-reversed and TTT-substituted FeuQ and FeuN mutant variants to test in our two-hybrid system if these mutants were still capable of physical interaction with their wild-type partner proteins. The periplasmic domains of the FeuQ (ETE→TTT), (ETE→TTK) and (ETE→KTK) variants were fused to the T18 fragment and tested against unaltered FeuN::T25. Conversely, the FeuN (GRP→TTT), (KKV→TTT), (RPKK→EPKK) and (RPKK→RPKE) variants were fused to the T25 fragment and tested against unaltered FeuQ::T18. In all cases, the charge-altering changes abolished the interaction (Fig. 6).

**DISCUSSION**

Our data suggest that FeuN interacts directly with the periplasmic domain of FeuQ at a surface mediated in part by electrostatic interactions, and this binding event sends a signal through the plasma membrane that ultimately stimulates FeuQ phosphatase activity. There are several TCS auxiliary proteins (e.g. PII, KipI, LuxP, CpxP, MzrA, Sda) that are known to activate net SK phosphatase activity, but by different means. Some of these proteins appear to directly increase the rate of phosphatase activity, others directly inhibit autokinase and/or phosphotransfer activities, and still others interfere with SK ligand recognition (Gerken & Misra, 2010; Jacques et al., 2011; Neiditch et al., 2006; Pioszak & Ninfa, 2003a; Rowland et al., 2004; Wang et al., 1997; Zhou et al., 2011).

Our ability to construct multiple kinase-dead alleles of FeuQ has allowed us to study FeuQ phosphatase activity in isolation and to discern the role of FeuN in stimulating phosphatase activity. We were unable to perform the converse experiment by selectively eliminating phosphatase activity. In some sensor kinases it has been shown that within a conserved E/DxxT motif of the DHp domain, the Thr residue is critical for phosphatase activity (Huynh & Stewart, 2011; Willett & Kirby, 2012). FeuQ shares this conserved motif, but mutations to the conserved Thr did not give a phenotype consistent with eliminated phosphatase activity (data not shown).

To understand how a direct interaction between FeuN and FeuQ in the periplasm might influence cytoplasmic FeuQ functions, we performed both random and targeted mutagenesis on FeuQ, focusing our attention on periplasmic mutations. We discovered critical regions for FeuQ signal modulation that cluster to the N- and C-terminal ends of the periplasmic sensing domain (see Figs 3 and 4). These clusters may occupy the same three-dimensional space, creating a single surface for direct interaction with FeuN or other external signals. On closer analysis of this potential interface, our attention was drawn to conserved compatibly charged amino acid residues: a negatively charged ETE sequence on FeuQ and a positively charged RPKK sequence on FeuN. We predicted that if these charged residues were involved in a direct interaction then reversing their charge would disrupt the FeuN/Q interaction due to charge repulsion. In single charge-reverse experiments our prediction proved accurate: charge-reversed mutants showed a dramatic loss of FeuN/Q inhibition in our reporter assay, and showed a loss of interaction in the two-hybrid assay. Interestingly, the charge-reversed variants are not easily corrected by making ‘compensatory’ charge reversals on the partner protein (data not shown). This suggests that a specific charge on the appropriate protein (positive on FeuN and negative on FeuQ) is required for a functional regulatory interaction. The fact that the feuQ double charge-reverse (ETE→KTK) mutant becomes FeuN-mimicking highlights the possibility that these periplasmic residues mediate a cytoplasmic phosphatase–kinase switch.

We propose that the FeuN/FeuQ interaction causes a conformational cascade that influences the relative rate of phosphorylation and dephosphorylation of FeuP in the cytoplasm. Presumably, many of the residues that mediate
this cascade are highlighted in the mutant analysis summarized in this paper.

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REFERENCES


