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FeuN, a novel modulator of two-component signaling identified in *Sinorhizobium meliloti*

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Summary

Sinorhizobium meliloti is a nitrogen-fixing bacterial symbiont of alfalfa and related legumes. Symbiotic infection by *S. meliloti* requires an osmosensory two-component system composed of the response regulator FeuP and the sensor kinase FeuQ. The FeuPQ pathway positively regulates transcription of multiple genes including *ndvA*, which encodes the cyclic glucan exporter. Here we show that proper regulation of this signaling pathway is essential for cell viability. Without the small 83 amino-acid protein FeuN, *S. meliloti* cells are unable to grow, and this phenotype is dependent on the FeuPQ pathway. Using *Escherichia coli* as a heterologous system, we show that expression of *feuP* and *feuQ* leads to a dramatic increase in *ndvA* promoter activity, but that simultaneous expression of *feuN* abrogates this effect. Random mutagenesis of the *feuPQ* bicistron revealed a defined region of the FeuQ protein in and around its two predicted transmembrane domains that is required for FeuN-dependent signaling modulation. Marker enzyme fusion experiments indicate that most of the FeuN polypeptide is localized to the periplasm. Our data support a model in which FeuN interacts directly with FeuQ to attenuate phosphorylation of FeuP, and that without this activity, hyperactive signaling through FeuPQ results in cessation of growth or death.

Introduction

The communication of environmental information to the bacterial transcriptional apparatus is often mediated by two-component signaling systems. Two-component systems commonly consist of a membrane-bound sensor histidine kinase that communicates environmental cues, by phosphorylation, to a cognate intracellular response regulator (Gao & Stock, 2009). The phosphorylated response regulator typically functions to activate or repress the transcription of target genes by interacting directly with regulatory DNA elements and RNA polymerase. An emerging theme in two-component system research is that of complex regulatory architectures involving protein functions beyond a simple histidine kinase/response regulator pair. These auxiliary proteins can extend the flexibility of two-component systems by modulating the activity of the sensor kinase or the response regulator, by exposing DNA for response regulator binding, or by connecting one two-component system to another (Buelow & Raivio, 2009, Mitrophanov & Groisman, 2008, Szurmant *et al.*, 2008, Gerken *et al.*, 2009, Chen *et al.*, 2008).

In the nitrogen-fixing symbiotic bacterium *Sinorhizobium meliloti*, several two-component systems are known to control the regulation of symbiotically important functions (Ronson *et al.*, 1987, David *et al.*, 1988, Cheng & Walker, 1998, Griffitts *et al.*, 2008). We previously reported that the *S. meliloti* proteins FeuQ (a sensor histidine kinase) and FeuP (a response regulator) play a critical role in symbiotic infection by positively regulating expression of

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the cyclic glucan exporter NdvA (Fig. 1A). FeuPQ-mediated transcriptional activation of *ndvA* was shown to be stimulated by low extracellular osmolarity (Griffitts et al., 2008). Here we show that a third protein, termed FeuN, functions as a negative regulator of signaling through FeuPQ. Loss of *feuN* is lethal to *S. meliloti*. Using *E. coli* as a heterologous analytical system, we show that interactions between FeuN, FeuPQ, and the downstream target gene *ndvA* are likely to be direct. In this system, we characterize mutations in *feuN*, *feuP*, and *feuQ* that abolish the negative regulation on signaling imposed by FeuN, and we present biochemical models to account for these observations. Finally, we show that overexpression of a putative glycerophosphodiester diesterase enzyme in *S. meliloti* suppresses lethality in the $\Delta feuN$ mutant, and provide evidence for the mechanism of this suppression.

Results

FeuN negatively regulates the FeuPQ pathway

Examination of the genetic region encoding FeuP and FeuQ indicates that a third open reading frame (ORF), designated SMC00065 (called *feuN* hereafter), may be cotranscribed with *feuP* and *feuQ* (Fig. 1B). Interestingly, multiple alphaproteobacterial genera, including the pathogens *Agrobacterium*, *Brucella*, and *Bartonella*, have *feuN*-like ORFs that are conserved adjacent to and upstream of their respective orthologues of *feuP* and *feuQ* (Fig. 1C). Based on these observations, we hypothesized that *feuN* may be functionally connected to the FeuPQ two-component system. We attempted to mutate *feuN* by allele exchange using the *sacB*-containing plasmid pJQ200sk (Quandt & Hynes, 1993), but these attempts failed. With *feuN* on a covering plasmid (pJG309), however, the chromosomal copy of *feuN* could be deleted without difficulty. These results suggest an essential role for *feuN* in the cell. Considering that *feuP* and *feuQ* are not essential for cell viability (Griffitts et al., 2008), we tested the possibility that the FeuN protein acts as a negative regulator of signaling through FeuQ and FeuP, such that the lethality associated with loss of the *feuN* gene arises from over-activity of the FeuPQ signaling system. According to this model, loss of either *feuQ* or *feuP* would suppress the lethal phenotype associated with loss of *feuN*. In fact, *feuN* is easily deleted from a $\Delta feuQ$ strain, and as predicted, our attempts to re-introduce a plasmid-borne copy of *feuQ* into the $\Delta feuN \Delta feuQ$ strain were unsuccessful (Fig. 2A).

If FeuN functions as a negative regulator of FeuPQ signaling, one would predict that loss of FeuN would increase FeuPQ signaling, and conversely, that overexpression of FeuN would decrease FeuPQ signaling. Since loss of FeuN is lethal, we tested the effects of FeuN overexpression. To *S. meliloti* cells harboring a chromosomal *ndvA-lacZ* reporter, we added a low copy number plasmid expressing *feuN* from the *Salmonella* P_{trp} promoter, which is constitutive in *S. meliloti*. Compared to the vector-only control strain, *ndvA-lacZ* expression was approximately halved when *feuN* was overexpressed (Fig. 2B). This effect was observed in both high- and low-osmolarity conditions. This modest effect is not surprising, since the vector-only control strain still harbors a normal chromosomal copy of *feuN*.

FeuP, FeuQ, and FeuN function heterologously in *E. coli*

Thus far, a direct interaction between either the response regulator FeuP and the target gene *ndvA*, or between FeuN and the FeuPQ proteins, has not been demonstrated. We reasoned that if these three proteins act in a direct fashion to modulate transcription from the *ndvA* promoter, their activity should be demonstrable in *E. coli*, which does not harbor an orthologous signaling system. The data in Figure 3 show that the plasmid-based $P_{ndvA-lacZ}$ reporter fusion is inactive in *E. coli* cells unless the *feuPQ* bicistron is also expressed. It should be noted that robust expression of $P_{ndvA-lacZ}$ requires only basal expression of *feuPQ* from the P_{tac} promoter. For this reason, all *E. coli*-based assays in this work are

performed without isopropyl- β -thiogalactopyranoside (IPTG) induction. In a subsequent set of experiments (see Fig. 4), the system represented in Figure 3 was elaborated with a third arabinose-inducible expression vector for tightly controlled expression of *feuN*. In this system, we observed that induction of *feuN* expression with arabinose leads to a dramatic (25-fold) decrease in *feuPQ*-dependent expression of the P_{ndvA} -*lacZ* reporter (Fig. 4A). A P_{BAD} vector-only control experiment did not exhibit any arabinose-dependent change in P_{ndvA} -*lacZ* expression. Taken together, these experiments support the idea that FeuQ and FeuP directly regulate expression from P_{ndvA} , and that FeuN directly acts on FeuQ and/or FeuP as an inhibitory modulator.

The effect of FeuN is dependent on the sensor kinase FeuQ

We used genetic methods in our heterologous *E. coli* system to better understand how FeuN acts to negatively regulate FeuPQ-dependent *ndvA* expression. We previously showed that in *S. meliloti*, FeuP retains some residual capacity to activate transcription of *ndvA* in the absence of FeuQ (Griffitts et al., 2008). When FeuQ is removed from the *E. coli*-based system, we also observe a low level of FeuP-dependent P_{ndvA} -*lacZ* expression that is abolished when the FeuP phosphorylation site (aspartate at position 51) is changed to glutamate (Fig. 4A). Coexpression of FeuN in the absence of FeuQ has little effect on reporter activity (Fig. 4A), suggesting that FeuQ is involved in the inhibitory activity of FeuN. To further test the idea that FeuN requires FeuQ for negative regulation of target gene expression, the FeuP(D51E) inactive variant was randomly mutagenized, and we screened for constitutively active, non-phosphorylatable (FeuP*) variants. Two distinct variants (FeuP*1 and FeuP*3) from this screen were used for further analysis. Compared to the FeuP(D51E) parent clone, FeuP*1 has three alterations (E51G, T79S and I112V), and FeuP*3 also has three alterations (K87G, K140R, and H223Q). These constitutive FeuP variants, each with distinct alterations, were assayed (in the absence of FeuQ) for responsiveness to coexpression with FeuN. As with the wild-type protein, these constitutive FeuP variants responded minimally (at most a 1.09-fold decrease in reporter gene expression) to the presence of FeuN (Fig. 4A). These observations suggest that FeuN works by regulating the FeuQ-dependent phosphorylation of FeuP rather than regulating downstream events such as DNA binding or interactions with RNA polymerase.

In an assay system in which *E. coli* cells are grown on solid medium supplemented with X-gal (Fig. 4B), we observe that FeuQ cooperates with FeuN to negatively regulate target gene expression. In the absence of FeuQ, FeuP can generate moderately blue colonies (Panel B3), consistent with the quantitative data from both *S. meliloti* (Griffitts et al., 2008) and *E. coli* (Fig. 4A) suggesting a basal FeuP activity that is FeuQ-independent. When FeuQ is co-expressed with FeuP, colonies are markedly more blue (compare Panels B3 and B1), and this positive regulatory effect is reflected in the quantitative data (see Fig. 4A). However, upon expression of FeuN in the presence of FeuQ (Panel B2), colonies become almost completely white, which is not the case in Panel B4, where the only difference is that FeuQ is present in Panel B2 and absent in Panel B4. This observation shows that FeuQ plays a role in blocking expression of the reporter when FeuN is present. Thus, when FeuN is absent, FeuQ has an observable *positive* regulatory function, but when FeuN is present, FeuQ has an observable *negative* regulatory function.

Alterations of FeuQ abrogate modulation by FeuN

The evidence presented thus far is consistent with a direct interaction between FeuN and FeuQ to prevent *ndvA* transcription. To refine this model and determine amino acid residues in FeuQ (or possibly FeuP) that are required for FeuN function, we performed a genetic screen for mutants of either *feuP* or *feuQ* that cause *ndvA* transcription to become less sensitive to FeuN. The *feuPQ* bicistronic cassette was PCR-amplified with Taq polymerase

to yield a mutant library that was subsequently re-transformed into *E. coli* harboring the arabinose-inducible FeuN expression vector and the P_{ndvA} -*lacZ* reporter plasmid (Fig. 5A). Cells were plated on medium supplemented with X-gal and arabinose. Most of the resulting colonies were white, due to inhibition of reporter expression by FeuN. However, several colonies from this screen were dark blue, indicating that one or more mutations in the *feuPQ* bicistron caused the phosphorylation cascade to be insensitive (or less sensitive) to the inhibitory effect of FeuN. Sequencing of *feuPQ* from 28 such colonies revealed that missense mutations occurred far more frequently within the *feuQ* gene (56 in *feuQ* versus 3 in *feuP*). While many clones contained only a single *feuQ* missense mutation, the three clones with *feuP* missense mutations possessed at least one other missense mutation in *feuQ*. Taken together, these results point to *feuQ* as likely the only gene between the two that mutated to confer diminished sensitivity to FeuN. Most FeuQ alterations that give rise to this phenotype cluster in and around its two predicted transmembrane domains. Figure 5B shows five such alterations, and a sixth alteration (E346K) occurring in the cytoplasmic kinase/phosphatase domain.

Clones harboring the single *feuQ* mutations shown in Figure 5B were re-ligated into the P_{tac} vector and re-transformed into *E. coli* cells harboring plasmids for arabinose-inducible FeuN production and P_{ndvA} -*lacZ* expression. Quantitative assays show that all six FeuQ variants exhibit markedly reduced sensitivity to FeuN than the wild-type protein (Fig. 6A). The L6P, S14P, N188D, and E346K variants are still responsive to FeuN expression, but it is attenuated. The P198L and I192E variants display complete insensitivity to FeuN inhibition. Interestingly, proline-198, which is located just two residues from the second FeuQ transmembrane domain (on the presumptive cytoplasmic side), is highly conserved in homologous sensor kinases throughout the alphaproteobacteria, as well as in the well characterized PhoQ and EnvZ sensor kinase proteins from the gammaproteobacteria.

FeuN-insensitive FeuQ variants are lethal in *S. meliloti*

In experiments presented earlier, we found that *feuN* cannot be deleted in *S. meliloti* unless *feuQ* is first deleted. We proposed that the lethality upon loss of *feuN* (hereafter termed ' Δ *feuN*-lethality') may be due to over-activation of net phosphoryl transfer from FeuQ to the FeuP response regulator. Further, experiments in both *S. meliloti* and the surrogate *E. coli* system point to FeuN as a negatively acting modulator, whose effects may be diminished or abrogated by specific mutations in *feuQ*. According to our model for Δ *feuN*-lethality, we predicted that FeuN-insensitive alleles of *feuQ* discovered in the *E. coli* system should be deleterious to *S. meliloti*. To test this, we cloned the FeuN-insensitive *feuQ* alleles into pRF771 for constitutive expression. These constructs were then transferred into strain B122, which has functional *feuN* and *feuP*, but lacks its chromosomal copy of *feuQ*. While a plasmid-borne copy of wild-type *feuQ* can be successfully introduced into B122, all six *feuQ* alleles exhibiting diminished *feuN* sensitivity in the *E. coli* system fail to yield viable *S. meliloti* colonies (Fig. 6B). This result supports the notion that the critical function of FeuN in *S. meliloti* is to regulate FeuQ activity.

In conjunction with the experiments just described, we wished to test the prediction that *feuP* function is required for the lethality associated with FeuN-insensitive variants of FeuQ. To do this, *feuQ*(P198L) was cloned on its own (with a synthetic ribosome binding site) into pRF771, resulting in plasmid pJG480. As expected, this plasmid was not accommodated by strain B122 (*feuN*⁺*feuP*⁺ Δ *feuQ*), but was readily transferred to strain B119 (*feuN*⁺ Δ *feuP* *feuQ*⁺). The pRF771 vector control was accommodated equally by both strains (data not shown). These results demonstrate that the lethality associated with loss of FeuN responsiveness requires the action of the response regulator FeuP.

Periplasmic localization of FeuN

That several alterations in FeuQ abrogate the action of FeuN in a heterologous host suggests that FeuQ is a direct target of FeuN. Based on sequence similarity with well characterized sensor kinases (such as the *E. coli* proteins EnvZ and PhoQ) and in silico analysis of probable transmembrane domains, it is evident that FeuQ is a canonical sensor kinase with two transmembrane segments (TM1: residues 10-32; TM2: residues 174-196), a periplasmic domain (residues 33-173) and a cytoplasmic domain (residues 197-461). A FeuN-FeuQ interaction could therefore occur in either the periplasm or the cytoplasm, or possibly in the membrane. To determine the localization of FeuN, we created translational fusions of FeuN to either GFP or *E. coli* PhoA. It is well established that the GFP chromophore develops poorly in the bacterial periplasm, and conversely, that PhoA is inactive in the cytoplasm, due to insufficient disulfide bond formation (Hoffman & Wright, 1985, Feilmeier *et al.*, 2000). Using our *E. coli* system, PhoA lacking its native secretion signal (Δ ss-PhoA) was fused to the C-terminus of full-length FeuN (residues 1-83) or C-terminally truncated FeuN (residues 1-48). In both instances, PhoA was directed to the periplasm, as evidenced by blue colony color on plates supplemented with X-phos (Fig. 7A). In a complementary experiment, we found that a translational fusion of full-length FeuN to GFP did not exhibit fluorescence (Fig. 7B), again indicative of a periplasmic localization. These experiments suggest that at least a large portion of the FeuN polypeptide is directed to the periplasm, but we cannot yet conclude whether the N-terminus remains integrated in the inner membrane, or if the polypeptide is processed and released as a soluble periplasmic protein. In either case, the result is in agreement with a model in which FeuN directly interacts with FeuQ in the periplasm or in the inner membrane. It is interesting to note that FeuN contains two cysteine residues that are absolutely conserved in homologous proteins found in *Agrobacterium*, *Brucella*, and *Bartonella* (see Fig. 1C). In a screen for nonfunctional *feuN* point mutants, both of these cysteine residues proved to be critical for FeuN function (not shown), indicating a possible periplasmic disulfide bond in the FeuN protein.

Mutations that suppress Δ *feuN*-lethality

The cause of Δ *feuN*-lethality in *S. meliloti* is not clear. The simplest explanation is that loss of *feuN* leads to deleterious hyper-expression of one or more gene targets of the FeuP response regulator. We postulated that a screen for mutations that suppress Δ *feuN*-lethality could shed light on the cause of this phenotype. The minitransposon Tn5-110 (Griffitts & Long, 2008) was used to mutagenize the otherwise viable Δ *feuN* Δ *feuQ* strain, resulting in a complex library of approximately 200,000 transposants. Into this library, we conjugally transferred the *feuPQ*-expressing plasmid pJG195. Accommodation of this plasmid requires suppression of Δ *feuN*-lethality, and excludes the possibility of this suppression occurring by Tn5-110 disruption of either *feuP* or *feuQ* (since these are introduced on a plasmid after mutagenesis). Five colonies from this selection were isolated and the Tn5-110 insertion sites were identified by arbitrary-PCR (Knobloch *et al.*, 2003). These five clones represented three distinct intergenic insertion events (*ig7*, *ig8*, and *ig13*), all occurring within a single 150-bp segment upstream of the uncharacterized gene SMC02646. In all three cases, Tn5-110 was oriented such that the outward facing, highly expressed P_{trp} promoter was reading into SMC02646 (Fig. 8A). Transduction of the *ig13* lesion into the original Δ *feuN* Δ *feuQ* strain, followed by successful conjugal transfer of pJG195, suggested that it was the cause of the suppression of Δ *feuN*-lethality.

We have considered possible mechanisms by which the *ig7*, *ig13*, and *ig8* insertion events give rise to the suppressor phenotype described above. First, the insertions may cause SMC02646 overexpression that in turn leads to survival in the absence of *feuN*. Second, the suppressor phenotype may be caused by P_{trp} -mediated overexpression of a gene located downstream of SMC02646, such as SMC02647 (see Fig. 8A). Third, the insertions may

misregulate the expression of one or more genes on the opposite side of the intergenic region (for example, SMc02645). Finally, these insertions may not be intergenic after all, and may disrupt some unidentified ORF or RNA-encoding sequence, with this loss-of-function event causing the suppressor phenotype. We devised a test with the potential to support the first possibility and rule out the other three. The SMc02646 ORF was cloned into the pRF771 vector, which places the plasmid-borne copy of P_{trp} upstream of SMc02646. This plasmid (or a vector-only control) was transferred to a wild-type strain, and in this context *feuN* deletion was attempted. In the presence of the SMc02646 overexpressing plasmid, *feuN* was easily deleted by allele exchange mutagenesis, while *feuN* deletion was not possible in the presence of the pRF771 vector (data not shown). The evidence therefore points to SMc02646 overexpression as the primary mechanism available to the cell (aside from disruption of FeuPQ signaling) for ameliorating the $\Delta feuN$ -lethality phenotype.

SMc02646 encodes a predicted glycerophosphodiesterase enzyme, distantly related to the *E. coli* proteins UgpQ and GlpQ. This enzyme family is involved in the disassembly of phospholipid-derived head groups, yielding *sn*-glycerol 3-phosphate and one of several possible alcohols, depending on the identity of the head group (Lin, 1976, Santelli *et al.*, 2004). *E. coli* UgpQ and GlpQ differ in their subcellular localization, with UgpQ residing in the cytoplasm and GlpQ residing in the periplasm (Tomassen *et al.*, 1991). Computational analysis of the protein encoded by SMc02646 using the program SignalP (Bendtsen *et al.*, 2004) strongly predicts that it is secreted to the periplasm. It is not immediately clear how an increase in periplasmic glycerophosphodiesterase activity would suppress $\Delta feuN$ -lethality, but we reasoned that the suppression could occur in one of two general ways: i) the activity leads to a decrease in signaling strength through FeuQ and FeuP, or ii) the activity rescues cell viability in spite of increased FeuPQ signaling brought about by loss of FeuN. To test this, we measured expression from the plasmid-based P_{ndvA} -*lacZ* fusion under various genetic conditions, and under conditions of high- and low-osmolarity (Fig. 8B). Compared to wild-type control cells, the *ig13* strain exhibited approximately 10-fold reduction in P_{ndvA} -*lacZ* expression, and this reduction occurred in both osmotic conditions. Interestingly, removal of *feuN* from the *ig13* genetic background caused a ~3-fold rebound of P_{ndvA} -*lacZ* expression, but to levels still below those in the wild type. These data indicate that SMc02646 upregulation decreases signaling strength through the FeuQ-FeuP two-component system. The general mechanism by which SMc02646 overexpression suppresses $\Delta feuN$ -lethality is therefore most likely by countering the positive effect of *feuN* deficiency on FeuP phosphorylation.

Discussion

We have characterized a novel protein (FeuN) and its role in *S. meliloti* as a negative regulator of signaling through the FeuQ-FeuP two-component system. Because FeuN is essential for *S. meliloti* viability, *E. coli*, which does not appear to encode an orthologous system, was employed as a surrogate to genetically dissect this pathway. This approach allowed us to demonstrate that transcriptional activation at the *ndvA* promoter is likely to be directly controlled by the FeuP response regulator. Transcriptional activation by FeuP is greatly stimulated by co-expression of the sensor kinase FeuQ, most likely because FeuQ phosphorylates FeuP. Simultaneous expression of FeuN, FeuQ, and FeuP leads to very low levels of reporter gene expression, and the inhibitory effect of FeuN depends on functional FeuQ. Thus FeuQ can switch between positively and negatively regulating functions depending on the presence of FeuN. A model to account for this property draws from the frequent observation that a given sensor kinase can act as either a phosphodonator or a phosphatase on its cognate response regulator, depending on context (Stock *et al.*, 2000, Yoshida *et al.*, 2002, Kostakioti *et al.*, 2009). The genetic analysis reported here is

consistent with a model in which FeuN stimulates FeuQ to transition from a phosphodonor to a phosphatase state.

Because FeuN and FeuQ cooperate to inactivate FeuP in a heterologous system, we conclude that FeuN and FeuQ likely interact with each other directly. It is now widely presumed that sensor kinases like FeuQ form homodimers in which two transmembrane helices from each protomer contribute to a four-helix bundle (Goldberg *et al.*, 2008, Cheung & Hendrickson, 2009, Sevvana *et al.*, 2008, Casino *et al.*, 2009, Gao & Stock, 2009). This membrane-localized interface is thought to play a critical role in the transduction of external signals from the periplasmic receptor domain to the cytoplasmic enzymatic domain. Our data indicate that at least a portion of FeuN localizes to the periplasm, consistent with an interaction between FeuN and the periplasmic domain of FeuQ. ExoR is a *S. meliloti* protein that is not homologous to FeuN, but which plays a similar role as a periplasmic negative regulator of signaling through the ExoS-ChvI two-component system (Wells *et al.*, 2007, Chen *et al.*, 2008). Like FeuN, the precise mechanism of ExoR action has not yet been elucidated.

To gain further insight into the physical site of FeuN function, we performed a random mutagenesis on the bicistronic *feuPQ* region, and found that alterations that abolish sensitivity to FeuN occur within the FeuQ polypeptide, often in and around the two putative transmembrane regions. In our series of FeuN-desensitized variants of FeuQ, the most dramatic cases occurred by alteration of residues at the cytoplasmic edge of the second FeuQ transmembrane domain (see Figures 5 and 6). The proline residue at position 198 is particularly intriguing because it is highly conserved in FeuQ homologues, including PhoQ from *Salmonella enterica* and EnvZ from *E. coli*. In fact, this specific residue is a highly conserved feature in what is known as the HAMP domain, which is a signal-transducing module that is found in a broad range of regulatory proteins, including histidine kinases, adenylyl cyclases, chemoreceptors, and phosphatases (Dunin-Horkawicz & Lupas, 2010). The structural basis for signal transduction through HAMP domains is currently a topic of great interest, and this work highlights the critical role of this conserved proline in maintaining signaling fidelity in HAMP-containing systems. None of the FeuN-desensitized FeuQ variants presented here display alterations in the periplasmic domain, where we predict a direct physical interaction with FeuN could be taking place. However, many of the FeuN-desensitized *feuQ* clones from the screen are not characterized in detail here because they harbor multiple (two to six) missense mutations. Of these less well characterized clones, many include amino acid altering mutations in the periplasmic domain.

We attempted to find loss-of-function mutations that would suppress $\Delta feuN$ -lethality, supposing that these mutations would identify genes most directly responsible for the lethality. In a screen that theoretically would have yielded such mutations, we only found transposon insertions that presumably lead to overexpression of SMc02646. This previously uncharacterized gene encodes a putative periplasmic glycerophosphodiesterase, belonging to an enzyme family involved in degrading phospholipid-derived glycerophosphodiesters into glycerol-3-phosphate and the corresponding alcohol, which may be choline, ethanolamine, inositol, glycerol, and so on. At present we can only speculate on the precise mechanism by which an increase in this enzymatic activity could suppress $\Delta feuN$ -lethality. It is clear that SMc02646 overexpression has the effect of impeding FeuPQ target gene expression, which has heretofore been observed to be modulated by osmotic conditions. It is therefore possible that increased periplasmic glycerophosphodiester hydrolysis increases the periplasmic osmolarity detected by FeuQ, leading to a negative effect on signaling that counters the deleteriously positive effect of *feuN* deficiency. Alternatively, FeuQ may respond to a specific product of SMc02646 enzyme activity, and not to osmolarity *per se*.

We previously reported that FeuQ-FeuP signaling is inversely proportional to external osmolarity, and we presented data suggesting that FeuQ is critical for the osmosensory property of the system (Griffitts et al., 2008). We have explored the idea that FeuN may also be involved in the osmosensory nature of this system, perhaps by physically disengaging from FeuQ under conditions of low osmolarity. The *ig13* suppressor mutation made it possible to remove *feuN* from the *S. meliloti* genome and further explore this model. The data in Figure 8 make it clear that FeuQ-FeuP signaling remains osmotically responsive even in the absence of *feuN*. We therefore conclude that FeuN, while affecting the overall magnitude of signaling, does not appear to be involved in sensing osmolarity.

It remains unclear how loss of *feuN* prevents growth of *S. meliloti* cells. One tempting model is based on the observation that FeuPQ signaling is dramatically enhanced under conditions of low osmolarity. Loss of FeuN may bring about a transcriptional response that might be appropriate for low-osmolarity conditions, but which may be lethal under conditions of moderate or high osmolarity. We are currently developing tools for recombinase-based, inducible gene deletion in *S. meliloti*, which should permit investigation into the cause of Δ *feuN*-lethality. With such a system, *feuN* could be deleted under specific conditions to establish whether osmolarity or some other external or internal (genetic) variables govern this intriguing phenotype.

With the completion of this genetic analysis, several aspects of the FeuNPQ “three-component” system remain to be explored, including the precise mode of interaction between FeuN and FeuQ, and more direct analysis of the effects of this interaction on FeuP phosphorylation status. It is also hoped that further study of this system will increase our understanding of the structural basis of signal transduction through transmembrane sensors, and reveal other general principles about the control of two-component signaling by both cytoplasmic and periplasmic auxiliary proteins. Finally, the degree to which the essential role of *feuN* is conserved in alphaproteobacterial pathogens, such as *Agrobacterium* and *Brucella*, could inform the development of narrow-spectrum antibacterial compounds.

Experimental Procedures

Bacterial culture and genetic techniques

Strains used are shown in Table 1. Unless otherwise specified, *E. coli* and *S. meliloti* were cultured in Luria-Bertani (LB) medium supplemented as appropriate with ampicillin (Ap, 100 µg/ml), L-arabinose (Ara, 0.3%), chloramphenicol (Cm, 30 µg/ml), gentamicin (Gm, 5 µg/ml for *E. coli* and 15 µg/ml for *S. meliloti*), kanamycin (Km, 30 µg/ml), neomycin (Nm, 80 µg/ml), streptomycin (Sm, 200 µg/ml), tetracycline (Tc, 10 µg/ml), sucrose (Suc, 40 g/l), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 50 µg/ml), and 5-bromo-4-chloro-3-indoxyl phosphate (X-phos, 50 µg/ml). Where culture osmolarity was manipulated, T4Y2 medium (4 g/l tryptone, 2 g/l yeast extract) was supplemented with 0 mM or 100 mM NaCl. Mobilization of plasmids from *E. coli* strain DH5α to *S. meliloti* was accomplished by overnight triparental mating on LB agar, with helper *E. coli* strain B001. Tn5-110 mini-transposon delivery, identification of transposon insertion sites by arbitrary-PCR, and phage-mediated transduction were described previously (Griffitts & Long, 2008).

Reporter-based assays

For β-galactosidase expression assays performed in *E. coli* and *S. meliloti*, bacterial growth and enzyme assays were carried out at 30°C. Stationary phase cultures were diluted 20-fold (*S. meliloti*) or 100-fold (*E. coli*), and allowed to reach exponential phase (OD₆₀₀~0.6) at which point assays were carried out according to (Miller, 1972). Alkaline phosphatase activity was observed qualitatively by growth on plates containing L-arabinose and X-phos.

For measurement of GFP fluorescence, cultures were first induced with L-arabinose for six hours. Following induction, 150 μ l of culture was transferred to a 96-well microtiter plate, and fluorescence was measured on a Packard Fusion fluorimeter with excitation wavelength of 460 and emission wavelength of 515/30.

Plasmid construction

PCR amplification was performed using Pfx50 (Invitrogen). Primer sequences are given in Table S1. To construct pJG298 for *feuN* deletion, the left-flanking region was amplified using oJG742 and oJG745. The right-flanking region was amplified using oJG744 and oJG743. These products were then fused by overlap-extension PCR using oJG742 and oJG743, followed by ligation into pJQ200sk. To construct pJG216, *feuQ* was amplified using oJG560 and oCF4, followed by ligation into pRF771. To construct pJG309, *feuN* was amplified using oJG768 and oJG769, followed by ligation into pRF771. To construct pJG395, SMC02646 was amplified using oJG977 and oJG978, followed by ligation into pRF771. To construct pJG195, *feuPQ* was amplified with oJG503 and oJG504, followed by ligation into pRF771. pJG249 was created by hybridizing oligos oJG641 and oJG642, followed by ligation into pMAL-c2g (New England Biolabs), which had been previously digested with NdeI and EcoRI. To construct pJG326, *feuPQ* was amplified with oJG815 and oJG817, followed by ligation into pJG249. To construct pJG329, *feuP* was amplified with oJG815 and oJG818 using pJG293 (Griffitts et al., 2008) as template, followed by ligation into pJG249. To construct pJG332, *feuP(D51E)* was amplified with oJG815 and oJG818 using pJG294 (Griffitts et al., 2008) as template, followed by ligation into pJG249. pJG351 was created by the ligation of three fragments: *kanR* from pJG110 (amplified with oJG848 and oJG849), *oriP15A* from pACYC177 (amplified with oJG846 and oJG847), and the *araC-P_{BAD}* region from *E. coli* MG1655 genomic DNA (amplified with oJG844 and oJG845). To construct pJG355, *feuN* was amplified using oJG869 and oJG870, followed by ligation into pJG351. To construct pJG433, *Δ ssphoA* was amplified from MG1655 genomic DNA using oRV006 and oJG964, followed by ligation into pJG351. To construct pJG387, full-length *phoA* was amplified from MG1655 genomic DNA using oJG961 and oJG964, followed by ligation into pJG351. To construct pJG437, a fragment encoding the first 40 amino acids of *E. coli* MG1655 *malE* was amplified using oRV004 and oRV005, followed by ligation into pJG351. To construct pJG438, a fragment encoding the first 50 amino acids of *E. coli* MG1655 *malE* was amplified using oRV003 and oRV004, followed by ligation into pJG351. To construct pJG435, a fragment encoding full-length *feuN* was amplified using oJG940 and oJG966, followed by ligation into pJG351. To construct pJG436, a fragment encoding the first 48 amino acids of *feuN* was amplified using oRV002 and oJG940, followed by ligation into pJG351. To construct pJG390, *gfpmut2* was amplified from pKEN-gfpmut2 (Cormack et al., 1996) using oJG944 and oJG943, followed by ligation into pJG351. To construct pJG391, *gfpmut2* was amplified from pKEN-gfpmut2 using oJG942 and oJG943, *feuN* was amplified from pJG355 using oJG940 and oJG941, and these two fragments were fused by overlap-extension PCR using oJG940 and oJG943, followed by ligation into pJG351. All constructs were confirmed by DNA sequencing.

Gene-specific mutagenesis

To create the *feuP*1* and *feuP*2* constitutive alleles, the non-phosphorylatable *feuP(D51E)* allele was amplified from pJG332 using primers oJG643 and oJG645, and *Taq* polymerase (New England Biolabs) according to manufacturer's specifications. The amplification product was ligated into expression vector pJG249. The ligation mixture was transformed into *E. coli* DH5 α /pJG286, followed by plating on LB-Tc/Ap/X-gal. Plasmid inserts from blue colonies (indicating constitutive FeuP activity) were re-ligated into pJG249, giving rise to pJG406 (*feuP*1*) and pJG408 (*feuP*2*), and *lacZ* expression was re-tested quantitatively, as shown in Figure 4.

For *feuPQ* mutagenesis, the *feuPQ* gene was amplified from pJG326 using primers oJG643 and oJG645 with *Taq* polymerase and the amplification product was ligated into expression vector pJG249. The ligation mixture was transformed into *E. coli* DH5 α /pJG286/pJG355, followed by plating on LB-Tc/Km/Ap/Ara/X-gal. Dark blue colonies (indicating loss of FeuN-mediated inhibition) were re-streaked and re-tested quantitatively. *feuPQ* mutants conferring the phenotype of interest were finally re-ligated into the same expression vector, giving rise to pC103, pC104, pC105, pC106, pC107, and pC108. These were re-transformed into the same recipient strain, followed by quantification of β -galactosidase activity as shown in Figure 6. For expression in *S. meliloti*, *feuPQ* inserts from pC104-pC108 were amplified with the high-fidelity polymerase Pfx50 (Invitrogen) using oJG503 and oJG504, followed by ligation into pRF771 giving rise to pJG419-pJG424 (see Table 1). The *S. meliloti* expression vector pJG480 was constructed by amplifying *feuQ*(P198L) from pC108 using oJG1147 and oJG1148, followed by ligation in to pRF771.

For *feuN* mutagenesis, the *feuN* segment was amplified with *Taq* polymerase from pJG355 using primers oJG915 and oJG931, followed by ligation into expression vector pJG351. The ligation was transformed into DH5 α /pJG286/pJG326, followed by plating on LB-Tc/Km/Ap/Ara/X-gal. Dark blue colonies (indicating loss of *feuN* function) were further characterized by re-plating and sequencing of mutant *feuN* inserts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *feuN* encodes a conserved alphaproteobacterial protein. A. Current model for the involvement of FeuQ and FeuP in sensing osmotic conditions and controlling cyclic glucan secretion. B. genetic position of *feuN* with respect to *feuP* and *feuQ*. C. Alignment of the FeuN polypeptide from *S. meliloti* (Sino) with orthologues encoded in *Agrobacterium tumefaciens* (Agro), *Brucella melitensis* (Bruc), and *Bartonella quintana* (Bart). Arrows indicate conserved cysteine residues.

Figure 2.

feun is essential in the *S. meliloti* 1021 wild-type genetic background. A. *feuQ* expression is lethal in a strain lacking *feun*. Plasmids pRF771 and pJG216 were transferred by triparental mating into recipients B122 ($\Delta feuQ$) and B584 ($\Delta feuN \Delta feuQ$) followed by selection on LB supplemented with streptomycin and tetracycline. B. To demonstrate that *FeuN* is a negative regulator, *ndvA-lacZ* reporter strain B304 harboring either pRF771 or *feun* overexpression plasmid pJG309 was assayed for *lacZ* expression after growth in T4Y2 medium with or without added NaCl. In this and subsequent figures, error bars indicate standard deviation from the mean in triplicate experiments. Grid lines have been added for easier discrimination of lower values.

Figure 3.

The FeuQ-FeuP pathway can be reconstituted in *E. coli*. A. The general arrangement of plasmids in tested strains. B. *lacZ* expression in strains harboring plasmid combinations. P_{tac} -(vector) denotes pJG249, P_{tac} -*feuPQ* denotes pJG326, (vector)-*lacZ* denotes pJG263, and P_{ndvA} -*lacZ* denotes pJG286. Note that P_{tac} β -galactoside inducer (IPTG) is not used in these experiments.

Figure 4.

FeuN acts directly on FeuQ-FeuP in a heterologous system. A. Inset illustrates the general arrangement of plasmids in tested strains. P_{BAD} expression plasmids include pJG351 (vector) and pJG355 (*feuN*); P_{tac} expression plasmids include pJG326 (*feuPQ*), pJG329 (*feuP*), pJG332 (*feuP(D51E)*), pJG406 (*feuP**1), and pJG408 (*feuP**3). Grid lines have been added for easier discrimination of lower values. B. *E. coli* strains were patched onto LB agar supplemented with X-gal and allowed to grow at 30°C for 2 days. All four strains harbor pJG286 and pJG355. The strain shown in B1 and B2 harbor pJG326, while the strain shown in B3 and B4 harbors pJG329. X-gal reaction is shown in the presence (+ara) or absence (-ara) of L-arabinose. Note that IPTG inducer of P_{tac} is not used in these experiments.

Figure 5.

Specific FeuQ alterations diminish the effects of FeuN. A. Schematic diagram of the mutant isolation strategy. B. Diagram of the FeuQ polypeptide (dark grey) sitting in the inner membrane (light grey) by way of two transmembrane domains (TM1 and TM2). Amino acid alterations giving rise to the FeuN-insensitivity phenotype are shown.

Figure 6.

FeuQ variants with reduced sensitivity to FeuN are deleterious to *S. meliloti*. A. *E. coli* strains containing reporter plasmid pJG286, *feuN* expression plasmid pJG355, and various *feuPQ* expression plasmids with different alleles of *feuQ* (see labels on figure) were assayed for β -galactosidase activity in the absence (–) or presence (+) of arabinose, which induces expression of *feuN*. As in previous experiments, the *feuPQ* cassette is expressed from the uninduced P_{tac} promoter. B. The same *feuPQ* alleles as in (A) were moved to the broad host range expression vector pRF771 and mated into *S. meliloti* strain B122 (Δ *feuQ*). Selection for transconjugants is shown after three days of incubation on LB plates containing streptomycin and tetracycline.

Figure 7.

FeuN is localized to the periplasm. A. Fusions to FeuN direct PhoA to the periplasm in *E. coli* DH5 α . Vector expressing PhoA lacking a secretion signal (Δ ss-PhoA) is shown as a negative control. Positive controls include full-length PhoA (FL-PhoA), PhoA with its native secretion signal replaced by the first 50 amino acids of MalE (MalE(1-50)- Δ ss-PhoA), and PhoA with its native secretion signal replaced by the first 40 amino acids of MalE (MalE(1-40)- Δ ss-PhoA). B. Fusion of FeuN to GFP abrogates fluorescence. Fluorescence measurements are described in Experimental Procedures.

Figure 8.

Overexpression of SMc02646 suppresses the Δ *feuN*-lethality phenotype in *S. meliloti*. **A.** Locations of Tn5-110 insertions that suppress Δ *feuN*-lethality are shown. The orientation of the constitutive *P_{trp}* promoter is indicated. **B.** The effect of *ig13* and *ig13* Δ *feuN* genotypes on FeuPQ-dependent *P_{ndvA}-lacZ* expression. Cultures were grown in 4/2 medium with (grey bars) and without (black bars) 100 mM NaCl. Grid lines have been added for easier discrimination of lower values.

Table 1

Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
DH5 α	<i>E. coli</i> cloning and expression strain	Grant <i>et al.</i> (1990)
B001	DH5 α harboring helper plasmid pRK600 (Cm ^R)	Finan <i>et al.</i> (1986)
Rm1021	<i>S. meliloti</i> wild-type progenitor to strains below (Sm ^R)	Meade <i>et al.</i> (1982)
B119	Δ <i>feuP</i>	Griffitts <i>et al.</i> (2008)
B122	Δ <i>feuQ</i>	Griffitts <i>et al.</i> (2008)
B304	P _{ndvA} - <i>lacZ</i>	Griffitts <i>et al.</i> (2008)
B511	pJG298 integrant	This study
B584	Δ <i>feuN</i> Δ <i>feuQ</i>	This study
B681	<i>ig13::Tn5-110</i>	This study
B689	Δ <i>feuN</i> <i>ig13::Tn5-110</i>	This study
Plasmids		
pJG110	Tn5-110 delivery vector (Ap ^R Km ^R /Nm ^R)	Griffitts and Long (2008)
pJQ200sk	Vector for construction of deletions (Suc ^S Gm ^R)	Quandt and Hynes (1993)
pJG298	pJQ200sk, <i>feuN</i> flanking segments	This study
pJG263	Promoterless <i>lacZ</i> (<i>ori</i> RK2; Tc ^R)	Griffitts <i>et al.</i> (2008)
pJG286	pJG263, P _{ndvA} - <i>lacZ</i>	Griffitts <i>et al.</i> (2008)
pRF771	Constitutive expression in <i>S. meliloti</i> (<i>ori</i> RK2; Tc ^R)	Wells and Long (2002)
pJG216	pRF771, <i>feuQ</i>	This study
pJG309	pRF771, <i>feuN</i>	This study
pJG395	pRF771, SMC02646	This study
pJG195	pRF771, <i>feuPQ</i>	This study
pJG419	pRF771, <i>feuPQ</i> (N188D)	This study
pJG420	pRF771, <i>feuPQ</i> (I192E)	This study
pJG421	pRF771, <i>feuPQ</i> (S14P)	This study
pJG422	pRF771, <i>feuPQ</i> (L6P)	This study
pJG423	pRF771, <i>feuPQ</i> (E346K)	This study
pJG424	pRF771, <i>feuPQ</i> (P198L)	This study
pJG480	pRF771, <i>feuQ</i> (P198L)	This study
pJG249	P _{tac} expression plasmid (<i>ori</i> ColE1; Ap ^R)	This study
pJG326	pJG249, P _{tac} - <i>feuPQ</i>	This study
pC103	pJG249, P _{tac} - <i>feuPQ</i> (N188D)	This study
pC104	pJG249, P _{tac} - <i>feuPQ</i> (I192E)	This study
pC105	pJG249, P _{tac} - <i>feuPQ</i> (S14P)	This study
pC106	pJG249, P _{tac} - <i>feuPQ</i> (L6P)	This study
pC107	pJG249, P _{tac} - <i>feuPQ</i> (E346K)	This study
pC108	pJG249, P _{tac} - <i>feuPQ</i> (P198L)	This study
pJG329	pJG249, P _{tac} - <i>feuP</i>	This study

Strain or plasmid	Relevant characteristics	Source or reference
pJG332	pJG249, $P_{lac-feuP(D51E)}$	This study
pJG406	pJG249, $P_{lac-feuP*1}$	This study
pJG408	pJG249, $P_{lac-feuP*3}$	This study
pJG351	P_{BAD} expression plasmid (<i>ori</i> p15A; Km ^R)	This study
pJG355	pJG351, $P_{BAD-feuN}$	This study
pJG433	pJG351, $P_{BAD-\Delta ssphoA}$	This study
pJG387	pJG351, P_{BAD} -full-length- <i>phoA</i>	This study
pJG437	pJG351, $P_{BAD-malE(1-40)-\Delta ssphoA}$	This study
pJG438	pJG351, $P_{BAD-malE(1-50)-\Delta ssphoA}$	This study
pJG435	pJG351, $P_{BAD-feuN(1-83)-\Delta ssphoA}$	This study
pJG436	pJG351, $P_{BAD-feuN(1-48)-\Delta ssphoA}$	This study
pJG390	pJG351, $P_{BAD-gfpmut2}$	This study
pJG391	pJG351, $P_{BAD-feuN(1-83)-gfpmut2}$	This study