Role of Sphingomonas sp. Strain Fr1 PhyR-NepR-\(\sigma^{EcfG}\) Cascade in General Stress Response and Identification of a Negative Regulator of PhyR\(^\dagger\)

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The general stress response in Alphaproteobacteria was recently described to depend on the alternative sigma factor \(\sigma^{Ecf}\); whose activity is regulated by its anti-sigma factor NepR. The response regulator PhyR, in turn, regulates NepR activity in a partner-switching mechanism according to which phosphorylation of PhyR triggers sequestration of NepR by the sigma factor-like effector domain of PhyR. Although genes encoding predicted histidine kinases can often be found associated with phyR, little is known about their role in modulation of PhyR phosphorylation status. We demonstrate here that the PhyR-NepR-\(\sigma^{EcfG}\) cascade is important for multiple stress resistance and competitiveness in the phyllosphere of a naturally abundant plant epiphyte, Sphingomonas sp. strain Fr1, and provide evidence that the partner switching mechanism is conserved. We furthermore identify a gene, designated phyP, encoding a predicted histidine kinase at the phyR locus as essential. Genetic epistasis experiments suggest that PhyP acts upstream of PhyR, keeping PhyR in an unphosphorylated, inactive state in nonstress conditions, strictly depending on the predicted phosphorylatable site of PhyP, His-341. In vitro experiments show that Escherichia coli inner membrane fractions containing PhyP disrupt the PhyR-P/NepR complex. Together with the fact that PhyP lacks an obvious ATPase domain, these results are in agreement with PhyP functioning as a phosphatase of PhyR, rather than a kinase.

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Little is known about signals perception and transduction to PhyR. Interaction between PhyR and NepR in vitro requires PhyR phosphorylation, and a phyR allele in which the phosphorylatable aspartate has been replaced by an alanine cannot complement a phyR mutant in the Alphaproteobacteria analyzed thus far (5, 11, 15, 20). In addition, the crystal structure of C. crescentus PhyR revealed that the receiver and sigma factor-like domains of unphosphorylated PhyR make tight contacts, and it was proposed that this closed conformation is disrupted upon PhyR phosphorylation (20). These data indicate that PhyR activity is controlled by phosphorylation, and thus one or several cognate PhyR histidine kinases should exist. In support of this hypothesis, histidine kinases are often found encoded at phyR loci in Alphaproteobacteria (47, 48). Although these proteins possess diverse signaling domains, which probably reflect the species-specific signals activating the system, their DHp (dimerization and histidine phosphotrans-
fer) domains remain conserved. Since this domain is the major determinant of specificity for partner recognition (7, 27, 46), these histidine kinases are prime candidates for functioning as cognate PhyR kinases. In agreement with this, it was recently shown that PhyK (CC_3474), the putative histidine kinase encoded at the phyR locus in *C. crescentus*, is required for phosphorylation of PhyR and activation of the cascade in response to stresses (29). However, no direct interaction between PhyK and PhyR was demonstrated in that study.

In the present study, we investigated the role and conservation of the PhyR-NepR-α^EcfG^ system in *Sphingomonas* sp. strain Fr1, a representative of the group of *Sphingomonas*-adaceae that comprises abundant epiphytes (9). In this organism, α^EcfG^, NepR, PhyR, and a putative histidine kinase are encoded at the same locus. The results demonstrate that the PhyR-NepR-α^EcfG^ cascade is involved in multiple stress resistance in *Sphingomonas* sp. Fr1 and that its mode of action is conserved. In addition, our data suggest that the putative histidine kinase encoded at the phyR locus does not function as a histidine kinase activating PhyR but rather acts as a phosphatase of PhyR. According to its proposed function, this protein was termed PhyP, for PhyR phosphatase.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The strains used in the present study are listed in Table 1. *Sphingomonas* sp. Fr1 strains were grown at 28°C in Luria broth (LB; Lennox), nutrient broth (NB), or minimal medium (MM [17]) containing 30 mM glucose. *E. coli* TOP10 (Invitrogen) or DH5α were used for cloning purposes, and *E. coli* BL21(DE3) was used for the production of recombinant proteins. *E. coli* strains were cultivated aerobically in LB medium at 37°C (TOP10 and DH5α), 30°C or 18°C (BL21[DE3]). When appropriate, the medium contained kanamycin (50 μg/ml), tetracycline (10 μg/ml), gentamicin (10 μg/ml), or carbenicillin (50 μg/ml).

**Plasmids and strain constructions.** Plasmids used in the present study are listed in Table 1. The oligonucleotides used to construct plasmids are listed in Table 1A in the supplemental material. All DNA manipulations were performed according to standard protocols (38). Phusin polymerase was used for all PCRs, and the restriction enzymes were from Fermentas. To construct plasmids for the PhyR-NepR-α^EcfG^ system in *Sphingomonas* sp. strain Fr1, a representative of the group of *Sphingomonas*-adaceae that comprises abundant epiphytes (9). In this organism, α^EcfG^, NepR, PhyR, and a putative histidine kinase are encoded at the same locus. The results demonstrate that the PhyR-NepR-α^EcfG^ cascade is involved in multiple stress resistance in *Sphingomonas* sp. Fr1 and that its mode of action is conserved. In addition, our data suggest that the putative histidine kinase encoded at the phyR locus does not function as a histidine kinase activating PhyR but rather acts as a phosphatase of PhyR. According to its proposed function, this protein was termed PhyP, for PhyR phosphatase.
| Strains or plasmid | Genotype and/or relevant features | Source or reference |
|-------------------|---------------------------------|---------------------|
| **E. coli** | F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR napG Δ(lacZYA-argF)U169 hsdR17 | Invitrogen |
| DH5α | | |
| TOP10 | F^- mcrA (par-HsdS-mcrBC) Δ80 lacZΔM15 Δ(lacX74) supE44 recA1 araD139 araC16 leu1-11,12 strA1 tryR proAB | Invitrogen |
| BL21(DE3) | F^- ompT gal dcm lon hsdS80r (DE3) lacI2ΔlacUV5-77 gene 1 ind1 sam7 nin5 | Invitrogen |
| BTH101 | F^- cyA99 araD139 galE15 galK16 rpsL1 hsdR2 mcrA1 mcrB1 | Euromedex |
| Sphingomonas sp. Fr1 | | |
| JVZ557 | Wild-type strain | 22 |
| JVZ1254 | ΔphyR | This study |
| JVZ1258 | ΔphyR ΔphyP::kan | This study |
| JVZ1286 | ΔecfG::kan | This study |
| JVZ1353 | ΔecfG::losP | This study |
| JVZ1357 | ΔphyR ΔphyP::losP | This study |
| JVZ1440 | ΔecfG::losP ΔphyP::kan | This study |
| JVZ1531 | ΔecfG::losP ΔecfR::losP | This study |
| JVZ1540 | ΔecfG::losP ΔecfR::losP | This study |
| JVZ1632 | ΔecfG::kan | This study |
| JVZ1647 | ΔecfG::losP | This study |
| **Plasmids** | | |
| pAK200 | Broad-host-range cloning plasmid with pBBR1 origin of replication and pK18 MCS; Km' | This study |
| pAK206 | pAK200 derivative; Gm' | This study |
| pAK206-NepR | pAK206 with nepR promoter and ORF; Gm' | This study |
| pAK206-PhyP | pAK206 with phyP promoter and ORF; Gm' | This study |
| pAK206-PhyP341A | pAK206 with phyP promoter and phyP(H341A) mutant allele; Gm' | This study |
| pAK400 | Ampicillin-sensitive pCM184 derivative; Km' Te' | This study |
| pAK400-ecfG2 | pAK400 with up- and downstream regions of ecfG2 flanking the kanamycin resistance cassette; Km' Te' | This study |
| pBBR1MCS-3 | Broad-host-range cloning vector; Te' | 26 |
| pCM157 | Cre recombinase expression plasmid; Te' | 32 |
| pCM184 | Allelic-exchange vector; Ap' Km' Te' | 32 |
| pCM184-ecfG | pCM184 with up- and downstream regions of ecfG flanking the kanamycin resistance cassette; Ap' Km' Te' | This study |
| pCM184-nepR | pCM184 with up- and downstream regions of nepR flanking the kanamycin resistance cassette; Ap' Km' Te' | This study |
| pCM184-phyP | pCM184 with up- and downstream regions of phyP flanking the kanamycin resistance cassette; Ap' Km' Te' | This study |
| pCM62 | Broad-host-range cloning vector; IncP origin of replication; Te' | 33 |
| pCM62-EcfG | pCM62 with ecfG ORF and 231 bp of upstream region; Te' | This study |
| pCM62-EcfGv2 | pCM62 with ecfG ORF and 284 bp of upstream region; Te' | This study |
| pCM62-PhyR | pCM62 with phyR promoter and ORF; Te' | This study |
| pCM62-PhyRD194A | pCM62 with phyR promoter and phyRD194A mutant allele; Te' | This study |
| pCR2.1-TOPO | TA cloning vector; Ap' Km' | This study |
| pDEST544 | Gateway cloning destination vector for fusions with a histidine-tagged NusA; Ap' | Addgene |
| pDEST544-EcfG | pDEST544 encoding a Histidine-NusA-EcfG fusion; Ap' | This study |
| pDsYFP | Source of sYFP2 | D. Bumann |
| pENTR4 | Entry vector for Gateway cloning; Cm' Km' | Invitrogen |
| pENTR4a-ecfG(N-thrombin) | pENTR4 with ecfG ORF and an N-terminal thrombin cleavage site; Km' | This study |
| pET24b | Expression vector for production of recombinant protein with a C-terminal His6 tag; Km' | Novagen |
| pET24b-PhyR | pET24b with phyR ORF; Km' | This study |
| pET26bII | Derivative of pET26b for expression of recombinant protein with an N-terminal thrombin or His6 tag; Km' | W. Malaga |
| pET26bII-NepR | pET26bII with nepR ORF; Km' | This study |
| pET26bII-PhyRN | pET26bII with the PhyR N-terminal sigma factor-like domain; Km' | This study |
| pKI8mobsacB | Broad-host-range sacB-based allelic-exchange vector; Km' | 40 |
| pKI8mobsacB-phyR | pKI8mobsacB with fused up- and downstream regions of phyR; Km' | This study |
| pKT25 | Low-copy-number plasmid for BACTH, encoding the Bordetella pertussis CyaA T2S fragment for fusions to its C terminus; Km' | Euromedex |
| pKT25-NepR | pKT25 with NepR; Km' | This study |
| pLM01 | Derivative of pCM62 without lac promoter; Km' | This study |
| pLM-sYFP2 | pLM01 with sYFP2 and RBS for transcriptional reporter fusions; Km' | This study |
| pLM-sYFP2-ecfG2p | pLM-sYFP2 derivative; EcfG2p-sYFP2 transcriptional reporter; Km' | This study |
| pUT18C | High-copy-number plasmid for BACTH, encoding the Bordetella pertussis CyaA T18 fragment for fusions to its C terminus; Ap' | Euromedex |
| pUT18C-EcfG | pUT18C with EcfG; Ap' | This study |
| pUT18C-EcfG2 | pUT18C with EcfG2; Ap' | This study |
| TOPO-PhyPH341A | pCR2.1-TOPO with phyP(H341A) allele; Ap' Km' | This study |

\[ a \] Cm', chloramphenicol resistance; Te', tetracycline resistance; Ap', ampicillin resistance; Gm', gentamicin resistance; Km', kanamycin resistance.
ends) was performed as described previously (13). The following primers were used for cDNA synthesis and PCR, respectively: ecfG3 R3 and ecfG4 R4, phyR R3 and phyR R4, nepR R2 and nepR R3, and phyP R5 and phyP R7.

Phenotypic assays. For all assays, bacteria were grown on NB supplemented with appropriate antibiotics. Precultures were inoculated with a small loop of bacteria from fresh NB plates and then grown for 8 to 10 h. The main cultures (20 ml in 100-m1 baffled flasks) were inoculated from precultures at an optical density at 600 nm (OD600) of 0.0005 to 0.001 and grown overnight at 28°C with orbital shaking at 220 rpm. Cultures at an OD600 of 1 were used for phenotypic assays.

For heat shock assays, the main cultures were transferred to a water bath at 46°C and incubated with shaking. Aliquots (100 ml) were removed after 20, 40, and 60 min of incubation, and then 10-fold serial dilutions were applied to NB plates. Aliquots taken before transfer to 46°C served as controls. For desiccation assays, 10-fold serial dilutions were spotted onto mixed cellulose ester filter membranes (HAWG-A0; Millipore), which were dried for 5 h under sterile airflow prior to transfer to NB plates. Control filter membranes were placed on NB plates immediately after spotting. For sucrose and sodium chloride stress assays, 10-fold serial dilutions of cultures were spotted onto NB plates containing 15% (wt/vol) sucrose or 300 mM NaCl, respectively, or NB plates (controls). Sensitivity to methylglyoxal was measured by disk-diffusion assays, as described previously (13). Sensitivity to 2% (vol/vol) methylglyoxal solution was wild.

Sensitivity to oxidizing agents was measured by disk diffusion assays using the following agents: 1 M hydrogen peroxide, 2.5 or 12.5% (wt/vol) paraquat (methyl viologen), 500 mM diamide, and 3% cumene hydroperoxide. Sensitivity to ultraviolet (UV) light was tested by exposing plates with 10-fold serial dilutions to 254-nm UV light for 30 s as described previously (13). CFU were counted or images were taken after 3 to 4 days of incubation at 28°C. Three biological replicates were performed for all experiments.

Seed sterilization, plant growth conditions, and harvest. Seeds of Arabidopsis thaliana ecotype Col-0 were sterilized as described previously (41). Seeds were inoculated with 5-fluorosulfuron (wild type or ΔeczG::kan mutant) or 1:1 mixtures of both strains, as described previously (42), except that glucose was used instead of succinate for bacterial cultures. Plants were grown at 28°C according to the method of Miller (34) with at least three biological replicates.

Phenotypic assays. The mapped promoters show homology to the pro-
strand appears to completely overlap the −35 box on the complementary strand.

**PhyR and αEcfG are involved in multiple stress resistance.** The PhyR-NepR-αEcfG cascade has been shown to control the general stress response in several Alphaproteobacteria (13, 15, 29, 31, 39). We thus examined whether this role is conserved in *Sphingomonas* sp. Fr1. Null mutants of phyR and ecfG, as well as their corresponding complemented strains, were constructed, and their sensitivity to different stresses was analyzed. The null mutants displayed the same growth rate as the wild-type strain under standard growth conditions, which indicated that neither protein is required for growth under optimal conditions (data not shown). Comparison of the ability to grow on high-osmolarity media (15% [wt/vol] sucrose or 300 mM NaCl) indicated that both mutants were more sensitive than the wild-type strain under standard growth conditions, which indicated that this strain can colonize plants. However, the null mutant was only slightly lower from plants inoculated with the wild-type (Fig. 2E). Therefore, while not essential for growth in planta under laboratory conditions, αEcfG confers a selective advantage in the phyllosphere.

Under the experimental conditions tested, no differences in sensitivity to hydrogen peroxide, cumene hydroperoxide, methyl viologen, UV light, ethanol, or the thiol-oxidizing agent diamide were observed between the wild-type and mutant strains (data not shown).

These findings indicate that PhyR and αEcfG are important for resistance against various stresses that are distinct in nature, including osmotic stress caused by high salt and sucrose, methylglyoxal, which attacks the nucleophilic centers of macromolecules, and elevated temperatures, which primarily cause mis- or unfolding of proteins. Since PhyR and αEcfG mediate resistance to multiple, unrelated stresses, these activities are referred to as a general stress response.

**αEcfG is important for plant colonization.** PhyR is essential for plant colonization by *M. extorquens* AM1 (14), which shows the importance of the general stress response in this harsh environment. Since *Sphingomonas* sp. Fr1 was isolated from the phyllosphere, the role of αEcfG in plant colonization was examined. *Arabidopsis thaliana* seeds were inoculated with wild-type and ecfG mutant strains, individually or in combination. In competition experiments, antibiotic resistance was used to distinguish wild-type from the kanamycin-resistant ecfG mutant strain. The average number of cells recovered from plants inoculated with the ecfG mutant was only slightly lower from plants inoculated with the wild-type (Fig. 2E), which indicates that this strain can colonize plants. However, when the ecfG mutant was tested for its capacity to compete with the wild type, only ca. 1% of recovered cells were ecfG mutants (Fig. 2E). Therefore, while not essential for growth in planta under laboratory conditions, αEcfG confers a selective advantage in the phyllosphere.
Protein interactions in the PhyR-NepR-\(\alpha^{EcfG}\) cascade are conserved in Sphingomonas sp. Fr1. The observation that phyR and ecfG mutants exhibit the same phenotype is in agreement with their function in the same regulatory cascade. To analyze conservation of the PhyR-NepR-\(\alpha^{EcfG}\) regulatory system further, we examined whether the protein interactions implicated in the proposed partner switching mechanism for Alphaproteobacteria (11) are conserved in Sphingomonas sp. Fr1, namely, whether NepR is the anti-sigma factor of \(\alpha^{EcfG}\) and PhyR the anti-anti-sigma factor. In vivo, elevated levels of nepR rendered the wild-type strain more sensitive to osmotic stress (Fig. 2F), which is a finding consistent with the role of NepR as a negative regulator of the PhyR-NepR-\(\alpha^{EcfG}\) cascade in other Alphaproteobacteria (11, 39). To assess the role of PhyR phosphorylation in Sphingomonas sp. Fr1 in vivo, the predicted phosphorylatable aspartate (residue 194) of PhyR was changed to an alanine. The ability of this mutant allele to complement a phyR mutant was then tested in phenotypic assays. As shown in Fig. 2, expression of phyR(D194A) in a \(\Delta\)phyR background failed to restore the wild-type phenotype, which suggests that this mutant protein, indeed, represents an inactive form of PhyR that cannot interact with NepR. Note that PhyR levels were similar in strains complemented with the wild-type or D194A phyR alleles, as assessed by immunoblot experiments with antibodies against PhyR (data not shown).

The interactions between NepR and \(\alpha^{EcfG}\) or PhyR in vitro were analyzed using size exclusion chromatography. PhyR and NepR were produced as histidine-tagged versions in E. coli, and \(\Delta\)ecfG as a fusion with histidine-tagged NusA, which was later removed by thrombin cleavage. Since \(\Delta\)ecfG tended to aggregate after cleavage, His-NusA-\(\Delta\)ecfG was incubated with NepR prior to cleavage and gel filtration experiments. \(\alpha^{EcfG}\) and NepR coeluted in a peak corresponding to an apparent molecular mass of 33 kDa (Fig. 3A), which is consistent with the formation of a heterodimer (theoretical molecular mass of 34.6 kDa), whereas NepR alone eluted with an apparent molecular mass of 23 kDa, a size consistent with a dimeric or trimeric form of NepR (predicted molecular mass of 8 kDa; data not shown). These findings indicate that NepR and \(\alpha^{EcfG}\) can form a complex, and both in vivo and in vitro data support the idea that NepR functions as an anti-sigma factor of \(\alpha^{EcfG}\) in Sphingomonas sp. Fr1.
Interactions between PhyR and NepR were examined by analytical gel filtration with acetyl phosphate used as the phospho-donor for PhyR phosphorylation. In the absence of acetyl phosphate, the two proteins eluted separately with apparent molecular masses of 23 and 39.9 kDa (predicted to be 30 kDa) for NepR and PhyR, respectively (Fig. 3B). In the presence of acetyl phosphate, equimolar amounts of PhyR and NepR eluted within a peak corresponding to 46.8 kDa, indicating heterodimer formation (predicted molecular mass of 38 kDa; Fig. 3C). The elution volume of PhyR was the same regardless of the presence of acetyl phosphate (see Fig. SA1A in the supplemental material), indicating that phosphorylated PhyR does not dimerize, similar to what has been observed for *M. extorquens* PhyR (11). Finally, we tested whether the ECF sigma factor-like domain of PhyR was sufficient for interactions with NepR. Alone, the ECF sigma factor-like domain of PhyR eluted with an apparent molecular mass of 26.5 kDa (predicted molecular mass of 15 kDa; data not shown). When applied in equimolar amounts, NepR and the ECF sigma factor-like domain of PhyR eluted in a single peak with an apparent molecular mass of 32 kDa (the theoretical molecular mass of the complex was 23 kDa; see Fig. SA1B in the supplemental material). Thus, only the phosphorylated form of PhyR interacts with NepR in vitro, and this occurs via its ECF sigma factor-like domain, as previously described for *M. extorquens* proteins (11).

Together with the interaction between NepR and σEcfG, these data demonstrate conservation of the mechanism underlying the PhyR-NepR-σEcfG cascade in *Sphingomonas* sp. Fr1 and other Alphaproteobacteria.

**Characterization of** σEcfG2. A second gene encoding a σEcfG2-type sigma factor, ecfG2, is predicted in the *Sphingomonas* sp. Fr1 draft genome (Fig. 1B) based on homology to σEcfG (43% identity, 61% similarity). To test whether this sigma factor might also be involved in the general stress response, the corresponding gene was deleted, and the resulting ecfG2 mutant subjected to phenotypic assays. No increased sensitivity to any of the stresses a phyR mutant is sensitive to was observed (data not shown). Bacterial two-hybrid assays based on *Bordetella pertussis* adenylate cyclase fragment complementation (24) further indicated that σEcfG2 does not interact with NepR, whereas σEcfG and NepR interacted in this system (Fig. 4A).

Finally, using transcriptional reporter fusions, it was demonstrated that the ecfG2 promoter is PhyR and σEcfG dependent (Fig. 4B), a finding consistent with the presence of a σEcfG-type promoter sequence in this region (Fig. 1B). These results suggest that σEcfG2 is not involved in the PhyR-NepR-σEcfG core cascade.

**PhyP is a negative regulator of the PhyR-NepR-σEcfG cascade.** The importance of PhyR phosphorylation in the partner switching model implies the existence of modulators of PhyR phosphorylation. Putative histidine kinases are encoded at phyR loci in a number of Alphaproteobacteria (13, 47, 48),

![FIG. 3. Interactions between NepR, σEcfG, and PhyR. Proteins were subjected to analytic gel filtration, analyzed by SDS-PAGE, and silver stained. (A) Complex formation between NepR and σEcfG. (B) No complex formation between NepR and PhyR is observed in the absence of acetyl phosphate. (C) In the presence of 25 mM acetyl phosphate, phosphorylated PhyR and NepR form a complex.](image)

![FIG. 4. Characterization of σEcfG2. (A) Bacterial two-hybrid analysis of interactions of NepR with σEcfG or σEcfG2. Interaction strength is reflected by the β-galactosidase activity, and values are given in Miller units as means ± the standard deviation of three independent experiments. pUT18C and pKT25 are empty control plasmids. (B) Activity of ecfG2 promoter transcriptional fusions to sYFP2 (pLM-sYFP2-ecfG2) or the empty control plasmid (pLM-sYFP2) in different genetic backgrounds. The expression of the reporter protein sYFP2 was measured by using IVIS Spectrum (Caliper Life Sciences) with 500-nm excitation and 540-nm emission filters. Fluorescence levels are represented in false color indicated by the scale bar on the right (in arbitrary units).](image)
including *Sphingomonas* sp. Fr1. These proteins are prime candidates for the control of PhyR phosphorylation and thus PhyR activation. All efforts to obtain a null mutant of the gene encoding the putative kinase at the *phyR* locus in *Sphingomonas* sp. Fr1 failed, suggesting that *phyP* might be essential. Interestingly, work in *S. melliloti* has shown that overactivation of the cascade is lethal, whereby *nepR* is essential and *ecfG* cannot be overexpressed unless co-overexpressed with *nepR* (39). By analogy, if overactivation of the cascade was lethal in *Sphingomonas* sp. Fr1 and if *phyP* was a negative regulator of the cascade, then a *phyP*-null mutation would also be lethal. The idea that PhyP functions as a negative regulator is based on the fact that (i) many histidine kinases are bifunctional, also acting on their cognate response regulators as phosphatases, and that (ii) deletion of these bifunctional kinases/phosphatases can lead to elevated levels of phosphorylated response regulator due to nonphysiological cross talk (27, 45).

To pursue the hypothesis that PhyP functions as a negative regulator of the cascade, we attempted to generate a *phyP* deletion in the Δ*phyR* or Δ*ecfG:*loxP::λ*P* genetic backgrounds. Both *phyR*-*phyP* and *ecfG-* *phyP* double mutants were readily obtained, which suggests that the apparent lethality of a *phyP* mutant is dependent on an intact PhyR-NepR-*ecfG* cascade and that PhyP acts upstream of PhyR, since *phyR* is epistatic to *phyP*. In accordance with this, when *phyR* or *ecfG* was provided in *trans* on a multicopy plasmid in the *phyR*-*phyP* or *ecfG*-*phyP* double mutant, respectively, no viable colonies were obtained even after 10 days of incubation, and the viability of these strains could be rescued by simultaneously expressing *phyP* in *trans*. To test whether *nepR* was also essential, a similar experiment was performed. An *ecfG* *nepR* double mutant (*ecfG:*loxP *nepR:*loxP) was constructed and transformed with a plasmid expressing *ecfG*. This strain was viable, although it showed a delayed growth phenotype, suggesting that *nepR* is not essential. In fact, it was also possible to obtain a nonpolar single *nepR* mutant that had a similar delayed growth phenotype. These results might be explained by the presence of a second, as-yet-unidentified *nepR* paralog in the *Sphingomonas* sp. Fr1 genome or another negative regulator of the PhyR cascade might, in fact, be lethal.

**Predicted phosphorylation sites in PhyR and PhyP are essential for PhyP activity.** If PhyP would keep PhyR in an inactive state, any condition mimicking this state should be viable in a *phyP* mutant background. Indeed, when the *phyR*-*phyP* mutant was transformed with a plasmid containing the *phyR*(D194A) allele, encoding a phosphorylation-incompetent PhyR, the resulting strain was viable. Similarly, coexpression of the wild-type *phyR* allele with *nepR* from a multicopy plasmid in the *phyR*-*phyP* mutant could rescue the lethal effect of the *phyR* allele, presumably because the elevated levels of NepR could titrate both active PhyR and *σ*<sub>EcFG</sub>. His-341 of PhyP corresponds to the conserved phosphorylatable histidine residue essential for kinase function in characterized histidine kinases. A H341A substitution in PhyP was constructed to study the role of this conserved residue. The resulting *phyP* allele, *phyP*(H341A), could not complement a *phyP*-*phyR* mutant coexpressing *phyR*, indicating that His-341 is important for PhyP function. Since *phyP* might encode a phosphatase and some bifunctional histidine kinases/phosphatases retain phosphatase activity when the conserved residue is replaced by certain amino acids (21, 23), the codon encoding His-341 of PhyP was randomized, and alleles were selected for that could restore viability in a *phyR*-*phyP* mutant coexpressing *phyR* (see Materials and Methods). However, of 11 alleles recovered after selection, all encoded histidine at position 341 (7 by the CAC and 4 by the original CAT codon). These results suggest that His-341 is essential for PhyP function, although we cannot rule out the possibility that some substitutions might preserve residual phosphatase activity (if such an activity exists) that, however, would be insufficient in *vivo*.

Altogether, these findings indicate that PhyP acts catalytically on PhyR, thereby preventing complex formation between NepR and PhyR, rather than by a titration mechanism like NepR. This is also consistent with the fact that PhyP overexpression, unlike NepR overexpression, does not lead to increased sensitivity to osmotic stress (Fig. 2F). One obvious explanation is that PhyP acts as a phosphatase of PhyR.

**PhyP disrupts the PhyR-P/NepR complex in vitro.** In order to test whether PhyP could disrupt the complex between phosphorylated PhyR (PhyR-P) and NepR in *vivo*, the PhyR-P/NepR complex was purified by gel filtration, thus removing acetyl phosphate, and incubated with the purified cytoplasmic part of PhyP. No disruption of the complex was observed, as assessed by analytic size exclusion chromatography (data not shown). Since the truncated form of PhyP might be inactive, full-length PhyP was tested for its capacity to disrupt the complex. PhyP and PhyP(H341A), respectively, were expressed in *E. coli*, and inner membrane fractions were prepared, followed by incubation with purified PhyR-P/NepR complex. For PhyP(H341A), no complex disruption was observed as judged by a peak elution volume of 8.91 ml compared to 8.68 ml for the purified PhyR-P/NepR complex and 9.37 ml for PhyR alone (data not shown). In contrast, wild-type PhyP apparently disrupted the PhyR-P/NepR complex, since the peak elution volume was 9.53 ml, close to the peak elution volume of 9.37 for PhyR alone (data not shown). To verify the presence of PhyR and/or NepR, elution fractions ranging from 7.8 to 10.6 ml were subjected to SDS-PAGE and Western blotting with antibodies against PhyR and NepR. As shown in Fig. 5, PhyR was present in both elution profiles, but NepR was present only in the one that had been incubated with the nonfunctional PhyP(H341A) protein, in which it coeluted with PhyR, thus confirming specific complex disruption by functional PhyP. Note that NepR was never observed in free form in these gel filtration experiments, which may be due to its hydrophobic character and in consequence its association with the membrane fraction (S. Campagne et al., unpublished data).

In summary, although we cannot exclude the unlikely possibility that a gene product conserved between distantly related *E. coli* and *Sphingomonas* sp. Fr1 mediates complex disruption of PhyR-P/NepR by PhyP, these results point toward a direct physical interaction of PhyP with PhyR, or the PhyR-P/NepR complex. Taken together, with the *in vivo* experiments, these results are in line with the hypothesis that PhyP acts as a phosphatase of PhyR.
DISCUSSION

The present study characterized the PhyR-NepR-σ^{EcfG} cascade in the epiphytic bacterium Sphingomonas sp. Fr1. In this organism, PhyR, NepR and σ^{EcfG} are encoded at the same locus, where a putative histidine kinase encoding gene, phyP, is also present. The phenotypic characterization of mutants determined that the PhyR-NepR-σ^{EcfG} cascade is an important factor in multiple stress resistance. In vitro experiments demonstrated that PhyR interacts with σ^{EcfG} and with phosphorylated PhyR. These results are in agreement with the in vivo data and together, they suggest conservation of the partner switching mechanism already proposed to occur in other Alphaproteobacteria (11). Epistasis experiments and mutant allelic were used to identify PhyP as a negative regulator of PhyR phosphorylation. Together with in vitro experiments, these data suggest that PhyP maintains PhyR in an unphosphorylated state in the absence of stress.

Besides conservation of the role of the PhyR-NepR-σ^{EcfG} cascade in multiple stress resistance, the system of Sphingomonas sp. Fr1 shares features observed in other Alphaproteobacteria. In Sphingomonas sp. Fr1, σ^{EcfG}-type promoters drive the expression of phyR, ecfG, and the nepR-phyP operon. This aspect seems conserved in other genera, although experimental evidence exists for only a few species (2, 15, 39, 48). However, other promoters probably exist, at least for phyR, since PhyR can still be detected by immunoblots in an ecfG mutant (data not shown). This finding is contrary to observations in B. japonicum and C. crescentus, where phyR expression appears solely dependent on σ^{EcfG} (15, 20). Another feature concerns the presence of a second σ^{EcfG}, σ^{ecfG2}, which apparently is not directly controlled by NepR through protein-protein interactions but instead regulated at the transcriptional level by σ^{EcfG}. This is reminiscent of the Caulobacter system, where two σ^{EcfG} proteins (SigT and SigU) exist. While the former regulates the latter, SigU does not appear to be a direct player in the partner switching mechanism but regulates a rather small subset of the SigT regulon (2). Whether the situation is similar in Sphingomonas sp. Fr1 necessitates further elucidation.

Regulation of PhyR phosphorylation is essential for system function according to the proposed model (Fig. 1C). Although our results suggest that PhyP acts as a phosphatase of PhyR, it remains to be shown whether PhyP also displays additional histidine kinase activity toward PhyR, similar to other bifunctional histidine kinases/phosphatases. However, bioinformatics analyses suggest that this is not the case. (i) Pfam or SMART (28, 43) do not predict any ATPase domain. (ii) No DUF or COG domains are predicted, which could be indicative of an as-yet-uncharacterized ATPase domain (10, 30). (iii) Multiple sequence alignments of phyR-associated kinases reveal no conservation of key residues in N, G1, and G2 box catalytic motifs in PhyP (16, 25) (see Fig. SA2 in the supplemental material). (iv) Finally, a BLASTp search with the PhyP part C-terminal to the DHp domain gave no significant hits except PhyP orthologs of related Sphingomonas species (data not shown). Altogether, this suggests that PhyP is devoid of ATPase activity rather than that it contains a novel or weakly conserved catalytic and ATPase (CA) domain. That a CA domain is not essential for the phosphatase activity of several bifunctional histidine kinases/phosphatases (6, 49) is in agreement with the proposed function of PhyP as a phosphatase of PhyR. In contrast, a CA domain is absolutely required for histidine kinase function, and this suggests that PhyP does not have kinase activity toward PhyR in addition.

How the PhyR response is triggered remains an open question. Our results indicate that lack of PhyP leads to activation of the PhyR-NepR-σ^{EcfG} cascade. In consequence, it is plausible to assume that turning down the phosphatase activity of PhyP is in principle sufficient to trigger the cascade and this might be one mechanism to activate the system. Consistent with the idea of PhyP activity being tunable, PhyP contains a putative periplasmic sensor domain and a linker HAMP domain typical for membrane-associated histidine kinases. However, it remains unknown how PhyR is phosphorylated, and additional modulators of PhyR phosphorylation are expected to exist, such as cognate sensor histidine kinases or endogenous small phospho-donors. Defining the complement of factors controlling PhyR phosphorylation and how they interact will be the subject of future work.

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