

# Sigma factor mimicry involved in regulation of general stress response

Anne Francez-Charlot<sup>1</sup>, Julia Frunzke<sup>1</sup>, Christian Reichen, Judith Zingg Ebnetter, Benjamin Gourion, and Julia A. Vorholt<sup>2</sup>

Institute of Microbiology, Eidgenössische Technische Hochschule Zurich, 8093 Zurich, Switzerland

Edited by Steven E. Lindow, University of California, Berkeley, CA, and approved January 7, 2009 (received for review October 13, 2008)

Bacteria have evolved regulatory traits to rapidly adapt to changing conditions. Two principal regulatory mechanisms to modulate gene expression consist of regulation via alternative sigma factors and phosphorylation-dependent response regulators. PhyR represents a recently discovered protein family combining parts of both systems: a sigma factor-like domain of the extracytoplasmic function (ECF) subfamily linked to a receiver domain of a response regulator. Here we investigated the mode of action of this key regulator of general stress response in *Methylobacterium extorquens*. Our results indicate that PhyR does not act as a genuine sigma factor but instead controls gene expression indirectly through protein-protein interactions. This is evident from the analysis of additional proteins involved in PhyR-dependent gene regulation. We demonstrated that the ECF sigma factor-like domain of PhyR interacts with a protein, designated NepR, upon phosphorylation of the PhyR receiver domain. Using transcriptome analysis and phenotypic assays, we showed that NepR is a negative regulator of PhyR response. Furthermore, we provide biochemical and genetic evidence that NepR exerts this inhibitory effect through sequestration of the ECF sigma factor  $\sigma^{\text{EcfG1}}$ . Our data support an unprecedented model according to which PhyR acts as a mimicry protein triggering a partner-switching mechanism. Such a regulation of general stress response clearly differs from the two known models operating via  $\sigma^S$  and  $\sigma^B$ . Given the absence of these master regulators and the concomitant conservation of PhyR in Alphaproteobacteria, the novel mechanism presented here is most likely central to the control of general stress response in this large subclass of Proteobacteria.

Alphaproteobacterium | two-component system | ECF sigma factor | PhyR

Bacterial responses to environmental stresses are mainly regulated at the level of transcription initiation (1). This is primarily carried out by alternative sigma factors, which allow promoter recognition and initiation of transcription as a subunit of RNA polymerase (2, 3). Among them, the large and diverse group of extracytoplasmic function (ECF) sigma factors plays a key role in adaptation to environmental conditions (4, 5). The activity of these ECF sigma factors is often negatively regulated by direct interaction with cognate anti-sigma factors, which prevent their association with the core RNA polymerase or facilitate holoenzyme dissociation (6–9).

Alternatively, regulation of gene expression is achieved by transcriptional regulators that bind DNA and modulate RNA polymerase holoenzyme recruitment at specific promoters (1). One large class of transcriptional regulators consists of response regulators of His-Asp phosphorelays (10–12). In classic systems, response regulators work together with a cognate sensor histidine kinase, and both proteins communicate via phosphorylation. Perception of a stimulus causes sensor autophosphorylation on a histidine residue; the phosphoryl group is then transferred to an aspartate residue of the response regulator, which modulates gene expression.

Both systems often result in the transduction of an external signal into the cytoplasm to regulate gene expression. The mechanisms from stimulus to gene regulation, however, are completely different in both systems. Interestingly, we recently identified an original regulator, PhyR, which combines domains of both systems: an

amino terminal ECF sigma factor-like domain and a carboxy terminal receiver domain of a response regulator. This raises the possibility that an ECF sigma factor could be directly regulated by phosphorylation (13). Our previous work indicated that PhyR is a key regulator of the general stress response in the methylophilic plant colonizer *Methylobacterium extorquens*. This protein is responsible for the activation of many stress-related genes, and a *phyR* deletion mutant shows drastic phenotypes when faced with various stressful conditions, including temperature shifts, oxidative stress, and desiccation (13, 14), which are all of relevance to the natural habitat of the bacteria (i.e., plant surfaces). Interestingly, PhyR is conserved in Alphaproteobacteria (13, 15), which altogether lack *rpoS* homologues, the gene encoding the well-known regulator of the general stress response first described in *Escherichia coli* (16, 17). This repartition may suggest that the phylogenetic group of Alphaproteobacteria uses PhyR to regulate the general stress response. How such a regulator activates stress genes is not known. Data from recent studies, however, provide indirect evidence for the involvement of additional proteins in a PhyR-dependent transduction cascade. Microsynteny is observed at the *phyR* locus of various Alphaproteobacteria: a small gene, *rmqI2793* (named *nepR*; see Results) in *M. extorquens*, is transcribed divergently to *phyR* (14). Except in *Methylobacterium spp.*, this gene precedes an ECF sigma factor-encoding gene. In *Sinorhizobium meliloti* and *Caulobacter crescentus*, both ECF sigma factors, RpoE2 and SigT, respectively, have been shown to be involved in the general stress response (18, 19). Genes regulated by RpoE2, SigT, and PhyR possess conserved ECF-type promoters (14, 18, 19). In addition, the product of the *nepR* homologue in *S. meliloti*, *smc1505*, was described as a negative regulator of the ECF sigma factor RpoE2 response (18). These findings suggest that these 3 proteins act in the same signal transduction cascade.

In the present work, we investigated how PhyR activates its target genes and the role of these additional proteins in the PhyR signal transduction cascade in *M. extorquens* using complementary genetic and biochemical approaches. Our findings suggest that upon activation, PhyR dissociates the sigma/anti-sigma interaction (i.e., the interaction between the anti-sigma factor NepR and the ECF sigma factor  $\sigma^{\text{EcfG1}}$ ). This mechanism represents the first example of the use of sigma factor mimicry in a partner-switching mechanism.

## Results

**Characterization of PhyR as a Response Regulator.** To characterize the mechanism of PhyR action, we first investigated whether PhyR was regulated by phosphorylation. Sequence analysis indicated that

Author contributions: A.F.-C., J.F., B.G., and J.A.V. designed research; A.F.-C., J.F., C.R., J.Z.E., and B.G. performed research; A.F.-C., J.F., C.R., J.Z.E., and B.G. analyzed data; and A.F.-C., J.F., and J.A.V. wrote the paper.

The authors declare no conflict of interest.

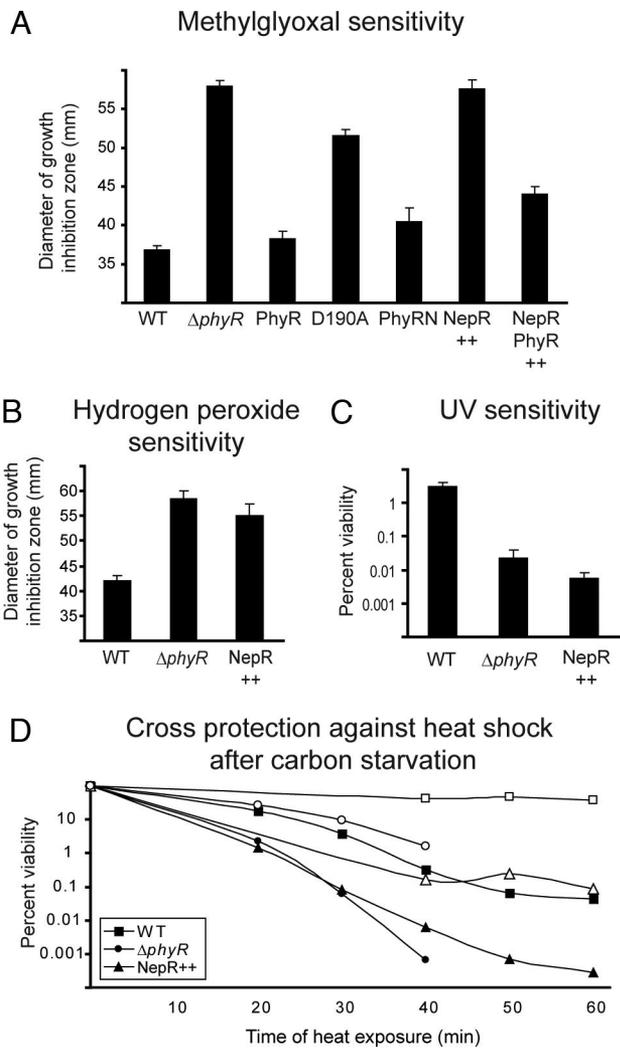
This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

<sup>1</sup>A.F.-C. and J.F. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed. E-mail: vorholt@micro.biol.ethz.ch.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0810291106/DCSupplemental](http://www.pnas.org/cgi/content/full/0810291106/DCSupplemental).



**Fig. 1.** Phenotypic analysis of *M. extorquens* mutant and overexpressing strains. (A and B) Methylglyoxal and hydrogen peroxide resistance. Sensitivity was tested in disk diffusion assays. Data indicate the diameter of the growth inhibition zone and are the mean of 3 measurements from at least 3 independent experiments. Bars show the standard error. (C) UV tolerance. UV tolerance was tested by spotting a dilution series of exponential-phase cultures on plates and exposing the plates to UV light (254 nm). Data indicate the percentage viability after 40 s of UV exposure compared with nonexposed cells and are the mean of 3 independent experiments. Bars show the standard error. (D) Cross-protection against heat shock after carbon starvation. Cultures were subjected to heat shock (closed symbols) or exposed to overnight carbon starvation before heat shock (open symbols). Data of 1 representative experiment out of 3 independent experiments is shown. Strains are noted as follows: WT, wild type;  $\Delta phyR$ ; PhyR,  $\Delta phyR/pCM62.phyR$ ; D190A,  $\Delta phyR/pCM62.phyRD190A$ ; PhyRN,  $\Delta phyR/pCM80.phyRNterm$ ; NepR++, wild type/pCM80.nepR (*rmq12793*); NepR PhyR++, wild type/pCM80.nepR.phyR.

the PhyR receiver domain possessed the 5 highly conserved amino acids that constitute the active site of described response regulators (10, 20): Glu-144, Asp-145, Asp-190, Thr-218, and Lys-238 (corresponding to Asp-12, Asp-13, Asp-57, Thr-87, and Lys-109, respectively, in CheY of *E. coli*), with Asp-190 being the predicted site of phosphorylation. We changed aspartate residue 190 to an alanine and tested whether this allele could complement a *phyR* mutant. For this purpose, *phyR* and *phyRD190A* were cloned into pCM62 under the control of its native promoter and the resulting plasmids introduced into the  $\Delta phyR$  mutant. Both strains were tested for their sensitivity to the electrophilic compound methylglyoxal in disk diffusion assays (14). As shown in Fig. 1A, expression of *phyRD190A*

in the  $\Delta phyR$  mutant did not restore the wild-type level of methylglyoxal resistance. This result suggests that phosphorylation of PhyR is required to activate the protein in vivo and that Asp-190 is the site of phosphorylation.

In some response regulators, the receiver domain inhibits the output domain, and this steric hindrance is suspended upon phosphorylation. Thus, such response regulators can be activated by removing their receiver domain (21–24). To test whether a similar mechanism was active for PhyR, we tested whether the N-terminal ECF sigma factor-like domain alone could complement a  $\Delta phyR$  mutant. To this end, a truncated PhyR protein containing the first 132 aa was overproduced in a *phyR* mutant, which was then tested for its sensitivity to methylglyoxal in disk diffusion assays. As shown in Fig. 1A, the ECF sigma factor-like domain of PhyR restored the wild-type phenotype, indicating that this domain is active in vivo. This finding suggests that upon phosphorylation, PhyR undergoes conformational changes that release the inhibitory effect of the receiver domain on the output domain.

**PhyR-Dependent Response Is Negatively Regulated by NepR.** The finding that PhyR is activated upon phosphorylation facilitates the study of the role of its output domain. We were unable to show a DNA-binding activity or an interaction with RNA polymerase of phosphorylated and unphosphorylated PhyR or of its N-terminal ECF sigma factor domain alone (data not shown). Therefore, we searched for other proteins that could be involved in the regulatory cascade directed by PhyR. As pointed out in the Introduction, *nepR* encodes a protein with unknown function and seemed to be a good candidate given that (i) it is conserved and found in synteny with *phyR* in Alphaproteobacteria (14), and (ii) the NepR homologue SMC01505 is a negative regulator of the ECF sigma factor RpoE2 in *S. meliloti* (18). Thus, this protein could represent a link between PhyR and another ECF sigma factor.

To analyze the role of this protein, a *nepR*-overexpressing strain was constructed and tested for its sensitivity to the broad spectrum of stresses that have been shown to affect a *phyR* mutant (14). As indicated in Fig. 1, this strain showed increased sensitivity to methylglyoxal (Fig. 1A), hydrogen peroxide (Fig. 1B), and UV exposure (Fig. 1C) compared with wild type. Furthermore, when exposed to drought stress for 3 days, the capacity of the *nepR*-overexpressing strain to form colonies was found to be 1,000-fold lower than wild type (data not shown). In addition, this strain was more sensitive to heat shock compared with wild type, and the protection against heat shock after carbon starvation seen in the wild type was partially lost when *nepR* was overexpressed (Fig. 1D). Thus, the phenotypes of the *phyR* mutant and the *nepR*-overexpressing strain were very similar, both qualitatively and quantitatively, involving the same stresses with comparable levels of sensitivity. These results suggest that NepR is a negative regulator of the PhyR-mediated response, and in light of this finding we named the protein NepR.

To confirm that PhyR and NepR regulate a common set of genes, we used microarray analyses to compare the transcriptional profile of the NepR-overexpressing strain with wild type and compared this regulon with the PhyR regulon, which we previously identified (14). Among 263 genes differentially regulated when NepR was overproduced, 114 were also part of the PhyR regulon (Table 1 and Table S1). These genes were found to be downregulated by NepR and upregulated by PhyR. This further supports the finding that NepR acts as a negative regulator of the PhyR response. Among the genes identified to be regulated by PhyR and NepR were several genes encoding typical stress proteins, including genes involved in oxidative stress (catalase), methylglyoxal resistance (lactoglutathione lyase), and heat shock response (RpoH) (Table 1). These findings are in agreement with the observed phenotypes.

Both proteins also had specific regulons. The 133 genes specific to PhyR mainly encoded proteins of unknown function or putative enzymes with unassigned pathways (Table S1). Among 149 genes

**Table 1. Genes of PhyR, NepR, and  $\sigma^{EcfG1}$  regulons\***

Function (gene homologue)	Gene	Fold change <i>phyR</i> OE/ $\Delta$ <i>phyR</i> <sup>†</sup>	Fold change <i>nepR</i> OE/WT <sup>†</sup>	Fold change $\Delta$ <i>ecfG1</i> /WT <sup>†</sup>
Detoxification/protection against stresses				
Catalase ( <i>katE</i> )	RMQ09549	10.2	-7.4	-7.9
Lactoylglutathione lyase ( <i>gloA</i> )	RMQ01575	4.9	-3.7	-2.1
Lactoylglutathione lyase ( <i>gloA</i> )	RMQ02894	6.1	-4.4	-2.7
Glutathione S-transferase ( <i>yqjG</i> )	RMQ06706	4.7	-2.2	-3.2
Glycogen debranching enzyme ( <i>glgX</i> )	RMQ05904	7.0	-4.2	-4.4
DNA protection during starvation protein ( <i>dps</i> )	RMQ05258	12.1	-7.7	-5.7
Regulation				
RNA polymerase $\sigma$ 32 factor ( <i>rpoH</i> )	RMQ12010	9.0	-4.3	-8.0
Transcriptional regulator CRP/FNR family	RMQ08139	24.6	-6.4	-10.2

OE, overexpression; WT, wild type.

\*Twenty-six additional genes are not presented in this table. For a complete list, see Table S1.

<sup>†</sup>Average changes of 3 experiments.

specific to the NepR regulon, we found genes encoding sulfur-oxidizing ability (25) and methanol oxidation to formaldehyde (26), as well as a region in the genome comprising approximately 50 genes, which seemed to correspond to a prophage (Table S1).

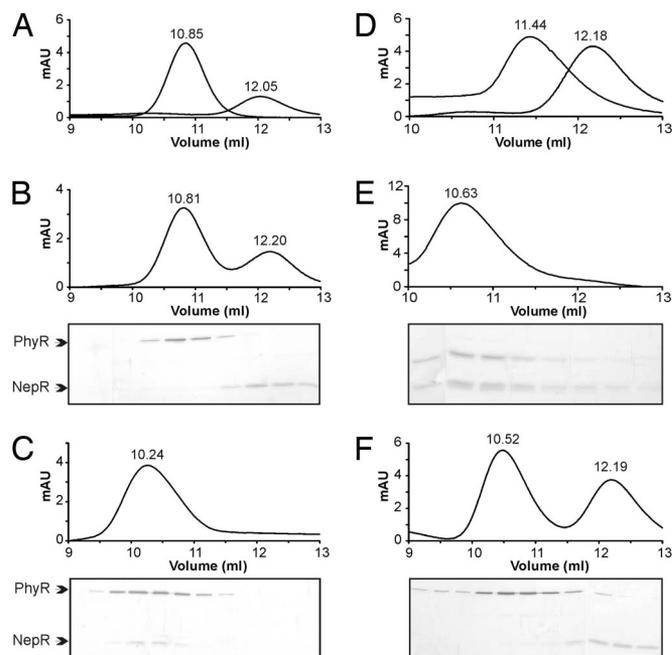
To further analyze whether PhyR and NepR belong to the same signaling cascade, we tested the possibility of suppressing the effects of *nepR* overexpression by coexpressing *nepR* and *phyR*. We constructed a pCM80.*nepR*.*phyR* plasmid, whereby both genes were under the control of the strong *mxoA* promoter, and introduced it in the wild-type strain. We then compared the methylglyoxal resistance of wild type, wild type/pCM80.*nepR*, and wild type/pCM80.*nepR*.*phyR*. As shown in Fig. 1A, the co-overexpression of *phyR* and *nepR* partially restored the wild-type phenotype relative to the overexpression of *nepR* alone. This finding clearly supports the hypothesis that both proteins act in the same signal transduction cascade.

#### Phosphorylated PhyR Interacts with NepR via Its ECF sigma Factor-Like Domain.

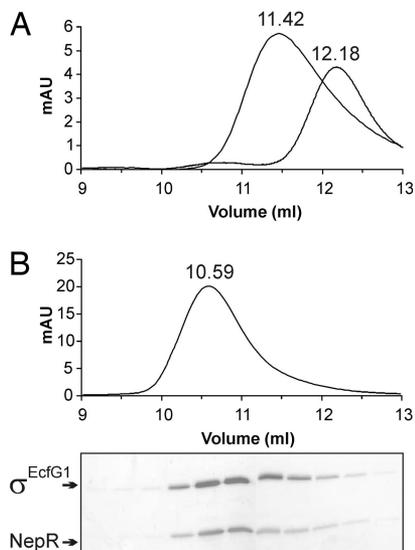
The functional relationship of PhyR and NepR could be explained by a physical interaction between the proteins. To test for the formation of such a complex in vitro, we performed analytic size-exclusion experiments. PhyR and NepR were overproduced in *E. coli* and purified by means of a histidine tag. When passed separately over a Superdex 75 10/300 column, the elution volume corresponded to an apparent molecular mass of PhyR of  $\approx$ 32 kDa (predicted molecular mass, 29.5 kDa), suggesting that it was present as a monomer. An identical elution profile was obtained with phosphorylated PhyR using acetyl phosphate as phospho-donor (data not shown). Thus it can be concluded that PhyR remains in its monomeric form when phosphorylated. Under the same conditions, the elution profile of purified NepR (predicted molecular mass, 11.8 kDa) revealed an apparent molecular mass of  $\approx$ 22 kDa, indicating homodimer formation (Fig. 2A). When nonphosphorylated PhyR was incubated with NepR, no complex formation between the 2 proteins was observed (Fig. 2B). PhyR phosphorylated by the addition of acetyl phosphate, however, directly interacted with NepR when both proteins were incubated in equimolar amounts before loading onto the column. The observed PhyR-P/NepR complex eluted with an apparent molecular mass of  $\approx$ 42 kDa, indicating the formation of a heterodimer composed of phosphorylated PhyR and NepR (Fig. 2C). When we tested the PhyRD190A mutant, no interaction with NepR was observed, with or without the addition of acetyl phosphate (Fig. 2F). These results clearly show that binding of PhyR to NepR strongly depends on the phosphorylation status of PhyR in vitro and are in agreement with results obtained in vivo.

To further characterize the interaction between PhyR and NepR, we tested whether the N-terminal ECF sigma factor domain of

PhyR alone could bind to NepR. As showed in Fig. 2D and E, when both proteins were incubated together, a heterodimeric complex of the N-terminal ECF sigma factor domain of PhyR (predicted mass, 18.9 kDa) and NepR was formed (apparent molecular mass of 35 kDa). This result indicates that the ECF sigma factor domain of PhyR is sufficient to interact with NepR. Altogether, these findings demonstrate that phosphorylation of PhyR enables interaction of its N-terminal ECF sigma factor-like domain with NepR.



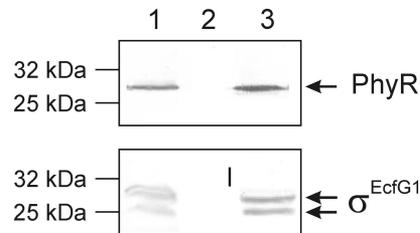
**Fig. 2.** Phosphorylated PhyR directly interacts with NepR via its N-terminal ECF sigma factor-like domain. Protein complexes were resolved on a Superdex 75 10/300 column (flow rate, 0.5 mL/min). The elution volume is indicated above the absorption peak. Fractions were subsequently analyzed on a 15% SDS-polyacrylamide gel and silver stained. (A) The elution profile of purified PhyR and NepR (each 3  $\mu$ M) passed separately over the column. (B) No interaction of PhyR and NepR was observed when incubated in equimolar amounts (each 3  $\mu$ M) in the absence of acetyl phosphate. (C) Upon the addition of acetyl phosphate to the binding buffer (25 mM), the formation of a heterodimeric complex of phosphorylated PhyR and NepR was observed. (D) Separate gel filtration of the purified N-terminal ECF sigma factor-like domain of PhyR and NepR. (E) The ECF domain of PhyR and NepR showed a direct interaction, even in the absence of acetyl phosphate. (F) A PhyR D190A mutant could not form a complex with NepR in the presence of 25 mM acetyl phosphate.



**Fig. 3.** NepR forms a complex with  $\sigma^{\text{EcfG1}}$ . (A) Elution profile of purified  $\sigma^{\text{EcfG1}}$  and NepR (4  $\mu\text{M}$ ) analyzed separately by gel filtration using a Superdex 75 10/300 column (flow rate, 0.5 mL/min). (B) Mixture of equimolar of each protein (8  $\mu\text{M}$  each) led to the formation of a heterodimeric complex of  $\sigma^{\text{EcfG1}}$  and NepR. Fractions were collected and analyzed on a 15% SDS-polyacrylamide gel and silver stained. The exact elution volume is indicated above the absorption peak.

**NepR Is an Anti-Sigma Factor of  $\sigma^{\text{EcfG1}}$ .** The finding that PhyR acts through protein-protein interactions raises the question of how the incoming signal is transferred from PhyR and NepR to the level of transcriptional regulation. As mentioned in the Introduction, *nepR* homologues are located upstream of genes encoding the ECF sigma factors RpoE2 and SigT in *S. meliloti* and *C. crescentus*, respectively. In fact, many genes regulated by RpoE2, SigT, and PhyR possess the same ECF sigma factor-type promoter (14, 18, 19). Although no gene encoding a putative ECF sigma factor could be detected in the *phyR* genomic region in *M. extorquens* AM1, the complete genome sequence indicated the presence of several genes encoding putative ECF sigma factors. Thus, we pursued the possibility that an ECF sigma factor could be responsible for PhyR-dependent stress gene regulation in this Alphaproteobacterium and that NepR might exert its function as an anti-sigma factor. Among 14 putative ECF sigma factors, RMQ08147 (designated  $\sigma^{\text{EcfG1}}$ , G standing for general stress response) exhibited the highest sequence identity to RpoE2 of *S. meliloti* (18) and was therefore considered to be a prime candidate for a possible involvement in the general stress response in *M. extorquens* AM1.

To address the question of whether NepR acts as an anti-sigma factor of  $\sigma^{\text{EcfG1}}$ , recombinant  $\sigma^{\text{EcfG1}}$  containing an N-terminal histidine tag was purified from *E. coli* to study potential interactions with NepR by gel filtration. No interaction of NepR and full-length  $\sigma^{\text{EcfG1}}$  was observed (data not shown). However, this could be due to solubility problems with purified  $\sigma^{\text{EcfG1}}$ , a problem that has been encountered for several sigma factors (27). In addition to the conserved regions 2 and 4 of sigma factors,  $\sigma^{\text{EcfG1}}$  possesses an unusual  $\approx 75$ -aa N-terminal extension. A truncated version of  $\sigma^{\text{EcfG1}}$  missing the first 64 aa showed better solubility and was used for interaction studies. When incubated in equimolar amounts, NepR and truncated  $\sigma^{\text{EcfG1}}$  formed a stable complex, which eluted from a Superdex 75 10/300 column corresponding to a molecular mass of 32 kDa, indicating heterodimer formation between NepR and  $\sigma^{\text{EcfG1}}$  (predicted molecular mass: NepR, 11.8 kDa; truncated version of  $\sigma^{\text{EcfG1}}$ , 21.5 kDa) (Fig. 3). When  $\sigma^{\text{EcfG1}}$ , full-length or truncated version, was incubated with PhyR or phosphorylated PhyR no complex formation was observed (data not shown).



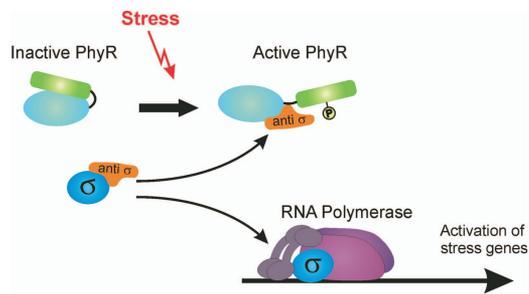
**Fig. 4.** NepR interacts in vivo with PhyR and  $\sigma^{\text{EcfG1}}$ . Recombinant NepR protein was overproduced in *M. extorquens* AM1/pCM80-nepRhis and purified by Ni-NTA via a C-terminal hexahistidine tag. Crude extract (lane 1), washing step (lane 2), and elution fraction (lane 3) were analyzed by Western blot using the polyclonal antibodies, anti-PhyR and anti- $\sigma^{\text{EcfG1}}$ .

To test for an interaction of NepR with PhyR and  $\sigma^{\text{EcfG1}}$  in vivo, NepR with a C-terminal histidine tag was overproduced in *M. extorquens* AM1/pCM80-nepRhis. When NepR and associated proteins were purified using Ni<sup>2+</sup>-activated agarose beads, both PhyR and  $\sigma^{\text{EcfG1}}$  were detected in the elution fraction using specific antibodies against PhyR and  $\sigma^{\text{EcfG1}}$ , respectively (Fig. 4). Neither PhyR nor  $\sigma^{\text{EcfG1}}$  were detected in the elution fraction for the negative control *M. extorquens* AM1/pCM80 (data not shown). In the case of  $\sigma^{\text{EcfG1}}$ , 2 specific bands were detected in Western blot analysis. N-terminal sequencing of the 2  $\sigma^{\text{EcfG1}}$  versions revealed that the upper band represented the full-length  $\sigma^{\text{EcfG1}}$  with the annotated N terminus MRNDT, whereas the smaller protein was missing 14 aa at the N terminus (N terminus: TDGRP). This finding indicates that NepR is able to interact with full-length  $\sigma^{\text{EcfG1}}$  in vivo.

To test the involvement of  $\sigma^{\text{EcfG1}}$  in the general stress response, we constructed an *ecfG1* deletion mutant and tested its sensitivity to stresses. No increased sensitivity was observed upon exposure to methylglyoxal, hydrogen peroxide, or UV light (data not shown). To identify the genes regulated by  $\sigma^{\text{EcfG1}}$ , we used microarray analyses. We found 39 genes differentially regulated in the *ecfG1* deletion mutant compared with wild type (Table S1); all but 1 were downregulated in the absence of *ecfG1*. A comparison of the PhyR, NepR, and  $\sigma^{\text{EcfG1}}$  regulons indicated that essentially all  $\sigma^{\text{EcfG1}}$ -regulated genes are part of the PhyR and/or NepR regulons (Table 1 and Table S1). As shown in Table 1, genes found in all 3 regulons include genes involved in protection against stresses and genes encoding putative transcriptional regulators. Taken together, these findings suggest that NepR acts as an anti-sigma factor of  $\sigma^{\text{EcfG1}}$ , the latter being responsible for the regulation of a subset of PhyR-regulated genes.

## Discussion

In the present study, we investigated the mechanism by which PhyR activates stress genes to trigger the general stress response in *M. extorquens*. Our results show that PhyR is a response regulator and is active in its phosphorylated form in vivo. Because no evidence of PhyR acting as a bona fide sigma factor was found, we investigated whether PhyR acts through protein-protein interaction and elucidated the role of 2 additional proteins in the regulatory pathway, NepR and  $\sigma^{\text{EcfG1}}$ . Our results indicate that the negative regulator of the general stress response NepR interacts with the ECF sigma factor  $\sigma^{\text{EcfG1}}$  and the ECF sigma factor-like domain of phosphorylated PhyR. Thus our results are in agreement with a partner-switching mechanism in which NepR represents the anti-sigma factor of  $\sigma^{\text{EcfG1}}$ , and PhyR represents the antagonist or anti-anti-sigma factor (Fig. 5). According to this model, PhyR is inactive in unstressed cells, and the ECF sigma factor  $\sigma^{\text{EcfG1}}$  is sequestered by its anti-sigma factor, NepR. In response to stress, PhyR is phosphorylated and interacts with NepR, thus releasing its interaction with  $\sigma^{\text{EcfG1}}$ , which can then associate with RNA polymerase to transcribe stress genes. Such a regulation involving a partner-switching mechanism to control the general stress response is



**Fig. 5.** Model of partner-switching mechanism controlling general stress response in *M. extorquens*. In unstressed cells, PhyR is inactive, and the ECF sigma factor  $\sigma^{\text{EcfG1}}$  is sequestered by its anti-sigma factor, NepR. In response to a stress, PhyR is phosphorylated and interacts with NepR, thus releasing  $\sigma^{\text{EcfG1}}$  and allowing  $\sigma^{\text{EcfG1}}$  to associate with RNA polymerase to transcribe stress genes. Note that additional  $\sigma^{\text{EcfG}}$  sigma factors are likely to be involved in PhyR dependent stress response (see *Discussion* for further details).

reminiscent of the system found in *Bacillus subtilis* (28). That system consists of a sigma factor, SigB, an anti-sigma factor, RsbW, and an antagonist protein, RsbV, with protein communication occurring via serine-threonine phosphorylation. RsbW phosphorylates RsbV in growing cells to maintain RsbV in an inactive state. In response to stimuli, 2 phosphatases, RsbP and RsbU, dephosphorylate RsbV, which can dissociate the RsbW-SigB complex. Both the *M. extorquens* and the *B. subtilis* regulatory systems use phosphorylation/dephosphorylation mechanisms of the antagonist protein to achieve the release of the sigma/anti-sigma interaction. However, whereas in the *B. subtilis* system the anti-sigma factor phosphorylates the antagonist protein, there is no indication for a kinase activity of NepR, because NepR has no homology to RsbW and does not contain a conserved kinase domain. Thus we expect that the phosphoryl group is provided to PhyR by one or more not-yet-identified histidine kinases. Genes encoding histidine kinases are often found associated to the *phyR* locus in Alphaproteobacteria, which is, however, not the case in *M. extorquens* (14). Here, we propose that the anti-anti-sigma factor PhyR uses a sigma factor mimicry mechanism to compete with  $\sigma^{\text{EcfG1}}$  for NepR binding. Although we cannot completely rule out that PhyR is a DNA-binding protein, sequence comparison of the PhyR sigma factor-like domain with ECF sigma factors suggests that this domain is a degenerate sigma factor, in which the region 2.4 involved in  $-10$  binding is lost and the  $-35$  binding region 4.2 is degenerate (Fig. S1; T. Mascher, unpublished data). Thus it may be assumed that only the determinants for the interaction with the anti-sigma factor are present, whereas those for DNA binding have been lost. Structure determination of NepR-PhyR and NepR- $\sigma^{\text{EcfG1}}$  complexes will be necessary to elucidate the binding sites of these interacting partners at the molecular level.

The next step in further characterizing this regulatory mechanism will be to demonstrate displacement of NepR from  $\sigma^{\text{EcfG1}}$  by phosphorylated PhyR. In preliminary experiments, only the NepR- $\sigma^{\text{EcfG1}}$  complex was formed when NepR,  $\sigma^{\text{EcfG1}}$ , and PhyR were incubated together, even in the presence of acetyl phosphate (data not shown). As mentioned in *Results*, however, we could only show the interaction of  $\sigma^{\text{EcfG1}}$  and NepR using the truncated form  $\sigma^{\text{EcfG1}}$   $\Delta 1-64$ . This truncated form apparently does not exist in vivo. The possibility that NepR interacts more strongly with  $\sigma^{\text{EcfG1}}$   $\Delta 1-64$  compared with the forms present in vivo could explain why PhyR cannot dissociate the complex. Surprisingly, this N-terminal extension shows similarity with NepR, leading to the hypothesis that some determinants for NepR binding in the  $\sigma^{\text{EcfG1}}$  structure are hindered by the N-terminal domain interacting with the rest of the protein. On the basis of this sequence identity, even a direct interaction of PhyR with  $\sigma^{\text{EcfG1}}$  is conceivable; however, this interaction was not observed in our in vitro studies. Again, structure

data will be required to answer these questions as well as complementation experiments with  $\sigma^{\text{EcfG1}}$  isoforms to understand the role of the N-terminal extension. In this context, it is interesting to note that although they are not typical, N-terminal extensions have been observed in other ECF sigma factors (29–31). In all of these studies, however, the role of the N-terminal extension has not yet been elucidated.

The observation that  $\sigma^{\text{EcfG1}}$  regulates only a subset of PhyR-dependent genes suggests that additional sigma factors are part of the regulatory pathway. In support of this idea, *M. extorquens* AM1 possesses 5 other sigma factors belonging to the same subgroup of ECF sigma factors (T. Mascher, personal communication) that could fulfill this function. Further investigation of the regulation of each sigma factor will provide important insights with respect to the specificity and redundancy of these ECF sigma factors in *M. extorquens*. The possible involvement of several ECF sigma factors raises the question of whether NepR is the anti-sigma for all of them or whether several anti-sigma factors are involved. The observation that approximately 90 genes are PhyR and NepR dependent but not regulated by  $\sigma^{\text{EcfG1}}$  suggests that NepR could control the activity of several sigma factors. In addition, the finding that specific regulons exist for PhyR and NepR supports the notion of a more complex regulatory network. The elucidation of this system together with the identification of proteins involved in the perception of activating stimuli will require more detailed analyses. Indeed, signal perception and transmission in the general stress response in the well-studied organisms *E. coli* and *B. subtilis* have proven to be highly complex (28, 32, 33).

In conclusion, central to the regulation of the general stress response in *M. extorquens* is the response regulator PhyR, which carries as its output domain a paralogue of an ECF sigma factor. This domain interacts with NepR, the anti-sigma factor of a bona fide sigma factor in a proposed model of partner switching. Such a core mechanism can be expected to exist in other Alphaproteobacteria as well. Thus, besides the well-described  $\sigma^{\text{S}}$ - and  $\sigma^{\text{B}}$ -dependent pathways, the PhyR-dependent cascade would represent a novel way to control general stress response in this large and diverse group of bacteria.

## Experimental Procedures

**Bacterial Strains and Growth Conditions.** Strains and plasmids used in this study are listed in Table S2. *M. extorquens* AM1 strains were grown at 28 °C on minimal medium (MM) (34) containing 18.5 mM sodium succinate and 120 mM methanol. *E. coli* DH5 $\alpha$  was used for all cloning purposes, and *E. coli* BL21 (DE3) was used for overproduction of recombinant proteins. *E. coli* strains were cultivated aerobically in LB medium at 37 °C (DH5 $\alpha$ ) or 30 °C [BL21(DE3)]. When appropriate, the media contained kanamycin (50  $\mu\text{g}/\text{mL}$ ), ampicillin (100  $\mu\text{g}/\text{mL}$ ), or tetracycline (10  $\mu\text{g}/\text{mL}$ ).

**Recombinant DNA Work.** The oligonucleotides used in this study are listed in Table S3 and were obtained from Microsynth. All DNA manipulations were performed according to standard protocols (35).

**Strain Constructions.** For complementation and overexpression studies in *M. extorquens* AM1, the different genes were introduced in plasmid pCM62 under the control of their own promoter or in its derivative pCM80, which contains the strong  $p_{\text{mxaF}}$  promoter (36).

To construct *ecfG1* deletion mutant, the broad-host-range *sacB*-based vector for unmarked allelic exchange pCM433 was used (37). Plasmids were introduced into *M. extorquens* AM1 by electroporation (38) or were transferred between *E. coli* S17 (39) and *M. extorquens* AM1 by conjugative transfer (40). Details of strain constructions can be found in *SI Experimental Procedures*.

**Phenotypic Assays.** For all assays, bacteria were grown at 28 °C in MM supplemented with methanol and succinate to an OD<sub>600</sub> of 1. All phenotypic assays were performed as described previously (14).

**RNA Sample Preparation and Transcriptome Analysis.** Overnight cultures of wild type/pCM80, wild type/pCM80-*nepR*, wild type, and  $\Delta\text{ecfG1}$  were diluted 1:10 in

fresh MM supplemented with methanol and succinate and grown to mid-exponential phase ( $OD_{600} = 1$ ). RNA was prepared as described previously (14).

Sixty-mer oligonucleotide microarrays of *M. extorquens* AM1 were described previously (41). Hybridization probe preparation, microarray hybridization, scanning, and basic data analyses were performed by MOGene. A significance analysis of microarrays [1 class, default settings, false discovery rate (FDR) 0.5% and FDR 2% for NepR and  $\sigma^{EcfG1}$  experiments, respectively] was performed on normalized data of 3 independent replicates. From this list, genes with a fold change average of at least 2.5 for NepR or 2 for  $\sigma^{EcfG1}$  and an initial *P* value  $<0.005$  for each replicate were considered to be significantly differentially expressed.

**Overproduction and Purification of PhyR, NepR, and  $\sigma^{EcfG1}$ .** The PhyR, NepR, and  $\sigma^{EcfG1}$  constructs were produced as His-tagged versions in *E. coli* BL21 (DE3) and purified using Ni-NTA and gel filtration. Detailed protein purification protocols can be found in *SI Experimental Procedures*. Purified PhyR, NepR, and  $\sigma^{EcfG1}$  were used to raise polyclonal antibodies from rabbits (Neoclone and Biogenes).

**Analytic Gel Filtration.** For complex formation, equimolar amounts of proteins (3  $\mu$ M) were mixed in binding buffer [50 mM Tris/HCl (pH 8.0), 40 mM KCl, 5 mM MgCl<sub>2</sub>, 5% glycerol, and 1 mM DTT] and incubated at room temperature for 10 min. For phosphorylation of PhyR, 25 mM acetyl phosphate was added to the incubation buffer. Subsequently, samples (total volume, 500  $\mu$ L) were loaded onto a Superdex 75 10/300 column (Amersham Pharmacia), equilibrated with buffer A [50 mM Tris/HCl (pH 8.0) and 150 mM NaCl] at a flow rate of 0.5 mL/min, using an Äkta purifier (Amersham Pharmacia). Apparent molecular masses were estimated by comparison to a low molecular mass standard (Amersham Pharma-

cia). After gel filtration, fractions were analyzed on a 15% SDS-polyacrylamide gel, and proteins were visualized by silver staining (42).

**In Vivo Interaction Studies.** For in vivo interaction studies, recombinant NepR containing a C-terminal hexahistidine tag was overproduced in *M. extorquens* AM1 using the pCM80-nepRhis vector (Table S2). *M. extorquens* AM1 transformed with an empty pCM80 vector was used as a negative control. Both strains were grown in 1 L of minimal medium containing succinate and methanol and harvested at an  $OD_{600}$  of 2.0. For cell extract preparation, cells were washed once and resuspended in 8 mL of TN15 buffer [20 mM Tris/HCl (pH 8.0), 150 mM NaCl, and 5 mM imidazol] supplemented with a protease inhibitor mixture (Complete, EDTA-free; Roche). Subsequently, the suspension was passed 3 times through a French pressure cell. After centrifugation (1 h, 4 °C, 100,000  $\times$  g), the supernatant was incubated with 0.5 mL of Ni-NTA agarose beads (Qiagen). The resin was then washed with 5 mL of TN15 buffer before eluting bound proteins with 3 mL of TN1200 buffer (TN15 with 200 mM imidazol). The fractions were separated on a 15% SDS-polyacrylamide gel, electrotransferred to a nitrocellulose membrane, and immunoblotted with anti-PhyR and anti- $\sigma^{EcfG1}$ . Bound antibody was visualized with an alkaline phosphatase-conjugated goat antirabbit secondary immunoglobulin (Bio-Rad).

**ACKNOWLEDGMENTS.** We thank Thorsten Mascher, University of Karlsruhe, for sharing unpublished data and for helpful discussion; and Nathalie Schmitt for technical assistance. This work was supported by the Swiss National Foundation, the European Molecular Biology Organization (fellowship to J.F.), and Eidgenössische Technische Hochschule Zurich.

- Browning DF, Busby SJ (2004) The regulation of bacterial transcription initiation. *Nat Rev Microbiol* 2:57–65.
- Murakami KS, Darst SA (2003) Bacterial RNA polymerases: The whole story. *Curr Opin Struct Biol* 13:31–39.
- Paget MS, Helmann JD (2003) The sigma70 family of sigma factors. *Genome Biol* 4:203.
- Helmann JD (2002) The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* 46:47–110.
- Butcher BG, Mascher T, Helmann JD (2008) in *Bacterial Physiology: A Molecular Approach*, ed El Sharoud W (Springer Verlag, Berlin), pp 233–261.
- Helmann JD (1999) Anti-sigma factors. *Curr Opin Microbiol* 2:135–141.
- Hughes KT, Mathee K (1998) The anti-sigma factors. *Annu Rev Microbiol* 52:231–286.
- Brooks BE, Buchanan SK (2008) Signaling mechanisms for activation of extracytoplasmic function (ECF) sigma factors. *Biochim Biophys Acta* 1778:1930–1945.
- Campbell EA, Westblade LF, Darst SA (2008) Regulation of bacterial RNA polymerase sigma factor activity: A structural perspective. *Curr Opin Microbiol* 11:121–127.
- Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. *Annu Rev Biochem* 69:183–215.
- West AH, Stock AM (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* 26:369–376.
- Laub MT, Goulian M (2007) Specificity in two-component signal transduction pathways. *Annu Rev Genet* 41:121–145.
- Gourion B, Rossignol M, Vorholt JA (2006) A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc Natl Acad Sci USA* 103:13186–13191.
- Gourion B, Francez-Charlot A, Vorholt JA (2008) PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. *J Bacteriol* 190:1027–1035.
- Galperin MY (2006) Structural classification of bacterial response regulators: Diversity of output domains and domain combinations. *J Bacteriol* 188:4169–4182.
- Lange R, Hengge-Aronis R (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* 5:49–59.
- McCann MP, Kidwell JP, Martin A (1991) The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *J Bacteriol* 173:4188–4194.
- Sauviac L, Philippe H, Phok K, Bruand C (2007) An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. *J Bacteriol* 189:4204–4216.
- Alvarez-Martinez CE, Lourenco RF, Baldini RL, Laub MT, Gomes SL (2007) The ECF sigma factor sigma(T) is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. *Mol Microbiol* 66:1240–1255.
- Lee SY, et al. (2001) Crystal structure of activated CheY. Comparison with other activated receiver domains. *J Biol Chem* 276:16425–16431.
- Grimsley JK, et al. (1994) Subunit composition and domain structure of the Spo0A sporulation transcription factor of *Bacillus subtilis*. *J Biol Chem* 269:16977–16982.
- Huala E, Stigter J, Ausubel FM (1992) The central domain of *Rhizobium leguminosarum* DctD functions independently to activate transcription. *J Bacteriol* 174:1428–1431.
- Kahn D, Ditta G (1991) Modular structure of FixJ: Homology of the transcriptional activator domain with the -35 binding domain of sigma factors. *Mol Microbiol* 5:987–997.
- Simms SA, Keane MG, Stock J (1985) Multiple forms of the CheB methyltransferase in bacterial chemotaxis. *J Biol Chem* 260:10161–10168.
- Friedrich CG, Bardischewsky F, Rother D, Quentmeier A, Fischer J (2005) Prokaryotic sulfur oxidation. *Curr Opin Microbiol* 8:253–259.
- Chistoserdova L, Chen SW, Lapidus A, Lidstrom ME (2003) Methylophily in *Methylobacterium extorquens* AM1 from a genomic point of view. *J Bacteriol* 185:2980–2987.
- Huang X, Decatur A, Sorokin A, Helmann JD (1997) The *Bacillus subtilis* sigma(X) protein is an extracytoplasmic function sigma factor contributing to survival at high temperature. *J Bacteriol* 179:2915–2921.
- Hecker M, Pane-Farre J, Volker U (2007) SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu Rev Microbiol* 61:215–236.
- Bibb MJ, Buttner MJ (2003) The *Streptomyces coelicolor* developmental transcription factor sigmaBldN is synthesized as a proprotein. *J Bacteriol* 185:2338–2345.
- Thakur KG, Joshi AM, Gopal B (2007) Structural and biophysical studies on two promoter recognition domains of the extra-cytoplasmic function sigma factor sigma(C) from *Mycobacterium tuberculosis*. *J Biol Chem* 282:4711–4718.
- Dona V, et al. (2008) Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor sigmaE in *Mycobacterium tuberculosis*. *J Bacteriol* 190:5963–5971.
- Bougourd A, Cunniff C, Baptiste PJ, Elliott T, Gottesman S (2008) Multiple pathways for regulation of sigmaS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. *Mol Microbiol* 68:298–313.
- Klauck E, Typas A, Hengge R (2007) The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. *Sci Prog* 90:103–127.
- Harder W, Attwood MM, Quayle JR (1973) Methanol assimilation by *Hyphomicrobium* sp. *J Gen Microbiol* 78:155–163.
- Sambrook J, Russell D (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), 3rd Ed.
- Marx CJ, Lidstrom ME (2001) Development of improved versatile broad-host-range vectors for use in methylophils and other Gram-negative bacteria. *Microbiology* 147:2065–2075.
- Marx CJ (2008) Development of a broad-host-range *sacB*-based vector for unmarked allelic exchange. *BMC Res Notes* 1:1.
- Toyama H, Anthony C, Lidstrom ME (1998) Construction of insertion and deletion *mx* mutants of *Methylobacterium extorquens* AM1 by electroporation. *FEMS Microbiol Lett* 166:1–7.
- Simon R, Priefer U, Puhler A (1983) A broad host range mobilization system for in vivo genetic-engineering—transposon mutagenesis in Gram-negative bacteria. *Bio-Technology* 1:784–791.
- Fulton GL, Nunn DN, Lidstrom ME (1984) Molecular cloning of a malyal coenzyme A lyase gene from *Pseudomonas* sp strain AM1, a facultative methylophil. *J Bacteriol* 160:718–723.
- Okubo Y, Skovran E, Guo X, Sivam D, Lidstrom ME (2007) Implementation of microarrays for *Methylobacterium extorquens* AM1. *Omic* 11:325–340.
- Nesterenko MV, Tilley M, Upton SJ (1994) A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J Biochem Biophys Methods* 28:239–242.