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The General Stress Response Factor EcfG Regulates Expression of the C-2 Hopanoid Methylase HpnP in *Rhodopseudomonas palustris* TIE-1

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Lipid molecules preserved in sedimentary rocks facilitate the reconstruction of events that have shaped the evolution of the Earth's biosphere. A key limitation for the interpretation of many of these molecular fossils is that their biological roles are still poorly understood. Here, we use *Rhodopseudomonas palustris* TIE-1 to identify factors that induce biosynthesis of 2-methyl hopanoids (2-MeBHPs), progenitors of 2-methyl hopanes, one of the most abundant biomarkers in the rock record. This is the first dissection of the regulation of *hpnP*, the gene encoding the C-2 hopanoid methylase, at the molecular level. We demonstrate that EcfG, the general stress response factor of alphaproteobacteria, regulates expression of *hpnP* under a variety of challenges, including high temperature, pH stress, and presence of nonionic osmolytes. Although higher *hpnP* transcription levels did not always result in higher amounts of total methylated hopanoids, the fraction of a particular kind of hopanoid, 2-methyl bacteriohopanetetrol, was consistently higher in the presence of most stressors in the wild type, but not in the Δ*ecfG* mutant, supporting a beneficial role for 2-MeBHPs in stress tolerance. The Δ*hpnP* mutant, however, did not exhibit a growth defect under the stress conditions tested except in acidic medium. This indicates that the inability to make 2-MeBHPs under most of these conditions can readily be compensated. Although stress is necessary to regulate 2-MeBHP production, the specific conditions under which 2-MeBHP biosynthesis is essential remain to be determined.

The most abundant molecular fossils on Earth are hopanes, diagenetic products of hopanoids (1, 2) (Fig. 1A). Hopanoids (or bacteriohopanepolyols [BHPs]) are steroid-like lipids with a C_{30} pentacyclic triterpenoid core that is often modified by methylation, unsaturation, and/or attachment to a C_5 ribose-derived side chain with a variety of substitutions (C_{35} or extended hopanoids) (3). Methylation is one of the few modifications that is geostable; thus, much attention has been paid to interpreting the meaning of C-2 or C-3 methyl hopanes in the rock record (Fig. 1A) (2). Classically, 2-methyl hopanes were viewed as proxies for cyanobacteria until 2-methyl hopanoids (2-MeBHPs) were found to be produced in equally high abundance by the anoxygenic phototroph *Rhodopseudomonas palustris* TIE-1 (4, 5). The list of potential 2-MeBHP producers broadened with the identification of the gene encoding the C-2 methylase HpnP in *R. palustris* TIE-1 (Fig. 1B). Through genomic and metagenomic analyses, it is now clear that the *hpnP* gene is absent in many cyanobacteria but is found in an acidobacterium and a monophyletic clade within the alphaproteobacteria (6); intriguingly, in most modern environments, alphaproteobacterial copies of *hpnP* dominate and are enriched in environments that support plant-microbe interactions (J. N. Ricci, M. L. Coleman, P. V. Welander, A. L. Sessions, R. E. Summons, J. R. Spear, and D. K. Newman, unpublished data). This raised the question: what is it about these environments that might elicit 2-MeBHP production?

Previously, we observed greater 2-MeBHP abundance in *R. palustris* in stationary phase or in response to pH stress (7). To test whether 2-MeBHPs are important for stress tolerance, we generated an *R. palustris hpnP* deletion mutant that is unable to synthesize 2-MeBHPs (6). However, using a bile salt and/or EDTA sensitivity assay we showed that absence of 2-MeBHPs does not significantly affect the permeability of either the outer or cytoplasmic membrane under chemoheterotrophic growth conditions, 30°C and pH 7 (8). Given the robustness of bacterial metabolism, it is often hard to find ecologically relevant conditions where a phenotype manifests. Furthermore, mutant phenotype(s) can be obscured by the presence of redundant pathways. As an alternative approach to gaining insight into what might stimulate 2-MeBHP production, we chose to explore stress-dependent regulation in *R. palustris* TIE-1.

Recent studies in a variety of alphaproteobacteria, including *Methylobacterium extorquens* AM1 (9, 10), *Sinorhizobium meliloti* 1021 (11, 12), *Bradyrhizobium japonicum* USDA 110 (13), *Caulobacter crescentus* CB15 (14–16), and *Sphingomonas* sp. strain Fr1 (17, 18), have established an extracellular function (ECF) sigma factor EcfG as the mediator of the general stress response (GSR) (Fig. 2A) (19). Under normal conditions, the activity of EcfG is kept in check by the anti-σ-factor NepR that binds to EcfG, thereby preventing its association with RNA polymerase (RNAP) to initiate gene transcription. On exposure to stress, a putative sensor histidine kinase (HK) phosphorylates the C-terminal receiver domain ($PhyR_{REC}$) of an anti anti-σ factor, PhyR, thus inducing a conformational change within the protein that exposes its N-terminal σ-like domain ($PhyR_{SL}$). NepR dissociates from EcfG to bind to the higher affinity $PhyR_{SL}$ domain, freeing EcfG to complex with RNAP and express stress-related genes. Noticing that *R. palustris* has a motif upstream of *hpnP* that could poten-

Received 13 February 2013 **Accepted** 18 March 2013
Published ahead of print 22 March 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00186-13.

doi:10.1128/JB.00186-13

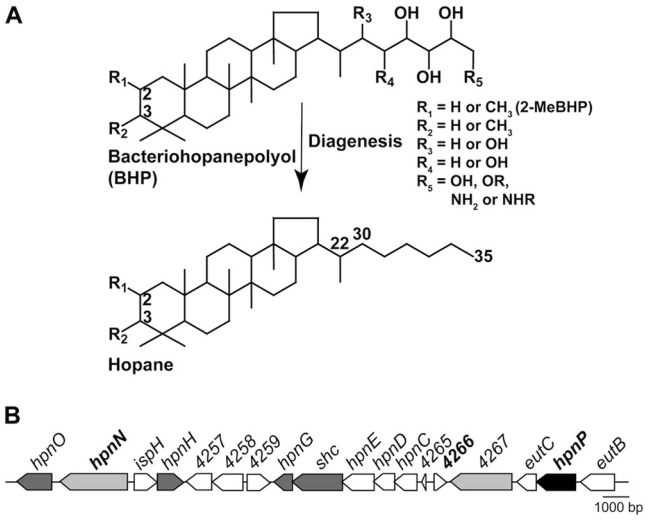
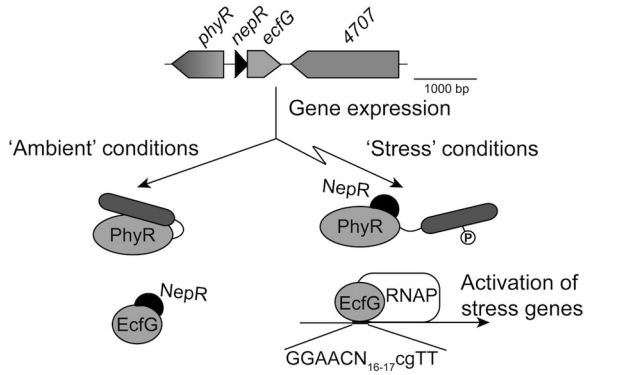


FIG 1 (A) Generalized structure of hopanoids (bacteriohopanepolyol [BHP]) and their diagenetic products hopanes. *R. palustris* TIE-1 produces the C_{30} hopanoids, diploptene (R_3 = H, with a C_{22}-C_{30} double bond, without the C_{31}-C_{35} tail) and diplopterol (R_3 = H, OH at C_{22}), as well as C_{35} or extended hopanoids, bacteriohopanetetrol (BHT; R_3, R_4 = H, R_5 = OH) and aminobacteriohopanetriol (R_3, R_4 = H, R_5 = NH_2), respectively. Diploptene, diplopterol, and BHT can be methylated at C_2 (2-MeBHP; R_1 = CH_3). (B) Putative hopanoid biosynthetic gene cluster identified in the *R. palustris* TIE-1 genome. The black arrow represents the gene under study that encodes the C-2 methylase HpnP. Dark gray arrows are genes known to be involved in hopanoid biosynthesis, and light gray arrows represent genes encoding hopanoid transporters. Putative EcfG-binding motifs are found upstream of *hpnN*, *Rpal_4266*, and *hpnP*.



B

Gene/Operon	Motif	Fold-regulation with heat shock (42°C/30°C)
nepR/ecfG	GGAACN$_{18}$TTN$_{80}$**GTG**	**11.4 ± 1.0/8.9 ± 1.0**
phyR	-	**10.4 ± 1.0**
Rpal_4707	GGAACN$_{19}$TTN$_{143}$**GTG**	**2.3 ± 0.2**
hpnN	GGAACN$_{17}$TTN$_{319}$**GTG**	1.0 ± 0.1
Rpal_4266	GGAACN$_{17}$TTN$_{111}$**ATG**	1.8 ± 0.2
hpnP	GGAACN$_{18}$TTN$_{94}$**ATG**	**7.5 ± 0.7**
shc	-	1.2 ± 0.1

FIG 2 (A) Regulatory model of the alphaproteobacterial general stress response (GSR) factor EcfG (adapted from reference 9). The genome locus encoding *R. palustris* TIE-1 GSR components is depicted at the top. Under ambient conditions, the anti-σ factor NepR binds EcfG, and the anti anti-σ factor PhyR is in an inactive unphosphorylated state. Upon exposure to stress, PhyR gets phosphorylated and binds to NepR, thus freeing EcfG to associate with RNA polymerase (RNAP) and bind to a consensus motif upstream of stress-related genes to initiate their transcription. Within the motif, the uppercase bases are well conserved, whereas the lowercase bases are not. N, any nucleotide base. (B) Expression of genes within the GSR and hopanoid biosynthesis loci under heat shock using qRT-PCR. Although all genes, except for *phyR*, within the GSR locus contain EcfG motif upstream of their start codons (indicated in boldface), only three genes within the hopanoid biosynthesis cluster possess this motif. In addition to these three, we also measured the expression of a fourth gene *shc* from this cluster. Each value represents the averages and standard errors of three biological replicates. A ≥2-fold upregulation in transcription was seen for all GSR genes and *hpnP* at 42°C versus 30°C (in boldface).

tially bind EcfG, we tested the hypothesis that *hpnP* expression in *R. palustris* is regulated by the GSR.

MATERIALS AND METHODS

Sequence analyses. The Integrated Microbial Genome (IMG) system (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) was used to access DNA and protein sequences, identify orthologs, and assess genomic context of genes (20). DNA and protein sequences were aligned with CLUSTAL W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In addition, CLUSTAL W2 was used to calculate pairwise sequence scores among orthologs, which are calculated as the number of identities between two sequences divided by the length of the alignment and are represented as a percentage. To search for the EcfG-binding consensus motif, upstream regions of genes upto 500 bp were extracted from *R. palustris* TIE1 genome. Regions overlapping adjacent genes were truncated at predicted gene boundaries. This data set was then used to find the GGAACN$_{18-19}$TT motif.

Bacterial strains, media, and growth conditions. The bacterial strains used in the present study are listed in Table 1. *Escherichia coli* strains were grown in lysogeny broth (LB) at 37°C. For aerobic chemoheterotrophic growth, *R. palustris* strains were grown in pH 7 medium containing 0.3% yeast extract, 0.3% peptone, and 50 mM morpholinepropanesulfonic acid (MOPS) with or without 5 mM succinate (YPMS or YPM, respectively) at 30°C in the dark with shaking at 250 rpm. Incubation temperature was changed to 4, 39, or 42°C when stated. To impose acidic (pH < 7) or alkaline stress (pH > 7), pH of the medium was modified by buffering with 100 mM MES (4-morpholineethanesulfonic acid) or 100 mM Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine], respectively. For hyperosmotic stress, ionic (1.5 or 3% NaCl) or nonionic (10% sucrose or 15% polyethylene glycol [PEG]) solutes were included in pH 7-buffered medium at the desired concentration. H_2O_2 was added to cultures to impose oxidative stress. For anaerobic phototrophic growth, *R. palustris* strains were grown in bicarbonate-buffered freshwater (FW) medium autotrophically with H_2/CO_2 (80/20%) at 5 lb/in^2 or heterotrophically with $N_2/CO_2/H_2$ (80/15/5%) at 5 lb/in^2 and 10 mM sodium acetate at 30°C in 50 W/m^2 light without shaking (21). Growth on solid medium was attained by adding 1.5% agar to LB, YPM, or YPMS. When necessary, gentamicin (Gm) was added to media at 20 μg/ml (*E. coli*) or 800 μg/ml (*R. palustris*).

For chemoheterotrophic growth curve assays, *R. palustris* strains were grown in YPM till exponential phase (optical density at 600 nm [OD_{600}] of ~0.15) (Spectronic 20D+; ThermoSpectronic). Then, 50 μl of each strain was used to inoculate 5-ml triplicate cultures in either regular YPM or YP in the presence of the stressor. Growth was monitored at OD_{600}. Similarly for phototrophic growth curves, 100 μl of OD_{660} ~0.5 cultures was used to inoculate 10-ml triplicate cultures, and growth was monitored at OD_{660}. For growth assays on solid medium, a 10-fold dilution series of saturated cultures of *R. palustris* strains was made using YPM. Next, 10 μl of the undiluted culture and each of the dilutions were spotted onto YP plates with or without stressor in triplicates using a multichannel pipettor. The plates were sealed with parafilm and incubated for at least 5 days to check growth.

DNA methods, plasmid construction, and transformation. All plasmid constructions and primers used in the present study are described in Table 1 and see Table S1 in the supplemental material. Standard methods

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype, description, and/or construction[a]	Source or reference
Strains		
DH10B	*E. coli*; F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* ϕ80*lacZ*ΔM15 *araD139* Δ(*ara leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$; used as a standard cloning strain	33
DKN379	*R. palustris* TIE-1; isolated as a phototrophic iron oxidizer from Woods Hole, MA; served as WT strain	21
DKN677	*R. palustris* TIE-1 Δ*shc*	7
DKN692	*R. palustris* TIE-1 Δ*hpnP*	6
DKN1216	*R. palustris* TIE-1 Δ*ecfG*; deletion of *Rpal_4706* in DKN379 using pGK202	This study
DKN1219	*R. palustris* TIE-1 Δ*phyR*; deletion of *Rpal_4704* in DKN379 using pGK199	This study
DKN1251	*R. palustris* TIE-1 *nepR* overexpression strain (P*lac-nepR*); DKN379 with pGK215	This study
DKN1285	*R. palustris* TIE-1 *phyR*(D189A); insertion of *phyR* site-directed mutant into DKN1219 using pGK214	This study
Plasmids		
pJQ200KS	Mobilizable suicide vector; *sacB*; Gm^r	23
pSRKGm	Complementation plasmid modified from pBBR1MCS-5; Gm^r	24
pGK199	SpeI-digested *phyR* upstream and downstream fusion PCR product amplified using the primers 4704upfor(2), 4704uprevfusion, 4704dnforfusion, and 4704dnrev and ligated to SpeI-digested pJQ200KS	This study
pGK202	SpeI-digested *ecfG* upstream and downstream fusion PCR product amplified using the primers 4706upfor(4), 4706uprevfusion, 4706dnforfusion, and 4706dnrev and ligated to SpeI-digested pJQ200KS	This study
pGK212	SpeI-digested *Rpal_4707* upstream and downstream fusion PCR product amplified using primers 4707upfor, 4707uprevfusion, 4707dnforfusion, and 4707dnrev and ligated to SpeI-digested pJQ200KS	This study
pGK214	SpeI-digested *phyR* upstream, coding (566th base mutated from A to C) and downstream fusion PCR product amplified using the primers 4704upfor(2), PhyRSDMcodrevw/fusion, PhyRSDMcodforw/fusion, and 4704dnrev and ligated to SpeI-digested pJQ200KS	This study
pGK215	NdeI/SpeI-digested *nepR* coding PCR product amplified using the primers 4705codfor and 4705codrev and ligated to pSRK-Gm	This study
pGK231	SpeI-digested *Rpal_4707* codon 347 upstream and downstream (1039th and 1040th base mutated from C to G and from A to C, respectively) fusion PCR product amplified using the primers hisKH347Aupfor, hisKH347Auprevfusion, hiskH347Adnforfusion, and hisKH347Adnrev and ligated to SpeI-digested pJQ200KS	This study

[a] Gm, gentamicin.

were used for plasmid DNA isolation and manipulation in *E. coli* (22). The DNA sequences for all cloning intermediates were confirmed by sequencing at Laragen (http://www.laragen.com/index.html) or Retrogen (http://sequencing.retrogen.com/). *R. palustris* strains were transformed using an Electroporator 2510 (Eppendorf, Hamburg, Germany) and then recovered in YPMS for at least 20 h. Subsequently, transformants were selected by plating different dilutions in the presence of Gm.

Construction of *R. palustris* deletion strains. The Gm selection and sucrose counterselection method described previously (7) was used to delete *ecfG* (DKN1216), *phyR* (DKN1219), or *Rpal_4707* using plasmids pGK202, pGK199, or pGK212, respectively, in the wild-type (WT) strain. DNA sequences immediately flanking the deleted genes were left intact to exclude the loss of regulatory elements needed for the expression of adjacent genes. To mutate residue 189 of PhyR from aspartate (D) to alanine (A), pGK214 was constructed by cloning the fusion PCR product of *phyR* 1 kb upstream, coding (the 566th base was mutated from A to C to change codon 189 from GAC to GCC) and 1-kb downstream regions in the suicide vector pJQ200KS (23). The site-directed *phyR*(D189A) mutant (DKN1285) was then isolated by the aforementioned selection/counterselection method in the Δ*phyR* (DKN1219) mutant background. The same method was attempted to mutate histidine (H) 347 of *Rpal_4707* to alanine (A) in WT. For this, pGK231 was constructed by cloning the fusion PCR product of 1-kb regions upstream and downstream of the modified codon 347 (CAC changed to GCC) into pJQ200KS. The *Rpal_4707* locus was then sequenced in segregants to check presence of the mutation. An *nepR* overexpression strain (DKN1251) was constructed by transforming pGK215, with an *nepR* coding region cloned downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter in pSRKGm (24), into the WT strain. The plasmid was maintained within the strain by growth in the presence of Gm and, when desired, *nepR* overexpression was induced by the addition of 1 mM IPTG.

Quantitative reverse-transcription PCR (qRT-PCR). All assays were performed with chemoheterotrophically grown aerobic cultures. For each assay, a YPM-grown log-phase culture (OD_{600} of ~0.15) of WT or mutant strain was used to start at least six 10-ml cultures with a 10^{-2} inoculum. At mid-exponential phase (OD_{600} of 0.10 to 0.15), triplicate cultures were exposed either to ambient (YPM, pH 7, 30°C) or stress conditions for 30 min. In the case of temperature stress, tubes were either incubated at 42 or 4°C. Oxidative stress was imposed by the addition of 2.8 μl of 3% H_2O_2 (250 μM). Stationary-phase cells were collected at an OD_{600} of ~0.35. For the rest of the stress conditions, the cells were first pelleted down by centrifugation at 5,000 × *g* for 20 min at 20°C and then exposed to the stressor by resuspension in 5 ml of YP-MES (pH 5), YP-Bicine (pH 9), YPM plus 1.5% NaCl, YPM plus 3% NaCl, YPM plus 10% sucrose, or YPM plus 15% PEG. All cultures underwent the same treatment with or without the stressor. At the end of the exposure time, cultures were transferred to 2 volumes of RNAprotect bacterial reagent (Qiagen, Valencia, CA), incubated for 5 min at room temperature, and centrifuged at 5,000 × *g* for 20 min at 4°C. RNA was extracted using the RNeasy minikit (Qiagen) with proteinase K and lysozyme treatment according to the manufacturer's instructions. Genomic DNA contamination was removed from each RNA sample with Turbo DNA-free DNase (Ambion, Foster City, CA). Then, 5 μl of a 20-ng/μl concentration of RNA was used in a 20-μl cDNA synthesis reaction using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Subsequently, 1 μl of cDNA was used as a template for qRT-PCR using the iTaq SYBR green Supermix with Rox (Bio-Rad) on the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). All samples were assayed in triplicate. Primer Express v2.0 (Applied Biosystems) or Primer3 software (http://frodo.wi.mit.edu/) was used to design gene-specific qRT-PCR primers (see Table S1 in the supplemental material), which were used at a final concentration of 200 nM in a total reaction volume of 20 μl. The cycling parameters used were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A final dissociation curve was determined for all reactions to ensure that a single product was amplified each time.

To generate a standard curve for quantification of each gene transcript (*nepR*, *ecfG*, *phyR*, *Rpal_4707*, *shc*, or *hpnP*) (7), a fragment of the gene was amplified using an invitroTCfor primer with a 5′ T7 promoter and an invitroTCrev primer (see Table S1 in the supplemental material). The PCR product was cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA), and 2 μl of the product was transcribed *in vitro* using a Megascript T7 kit (Ambion). In addition to other kit components, we added 2 μl of recombinant RNasin RNase inhibitor (Promega, Madison, WI) to the reaction mixture to inhibit any RNase activity during the 4-h incubation period.

The reaction was treated with 2 μl of Turbo DNA-free DNase for an hour to remove any DNA template and then further purified with a Megaclear kit (Ambion) to get rid of unincorporated nucleoside triphosphates, enzymes, and buffer components. The purity of the *in vitro* transcript was confirmed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA) at Millard and Muriel Jacobs Genetics and Genomics Laboratory, Caltech. The quantity of each transcript was determined by averaging triplicate measurements of its concentration on a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Assuming the average mass of a ribonucleotide to be 321 Da, the concentration of each transcript was converted to copy numbers/μl, diluted to 2×10^{10} copies/μl, and stored at −80°C. A standard curve was generated by performing two independent 10-fold serial dilutions of the RNA standard and using 5 μl of each dilution in the reverse transcription reaction. Then, 1 μl of the cDNA was used as a template for qRT-PCR using the gene-specific primers. The standard curve was generated by plotting threshold cycles (C_T) versus the log of the copy number. The C_T of each RNA sample was used to calculate the transcript copy number from linear regression of the standard curve.

Total hopanoid quantification in *R. palustris*. To quantify hopanoids after exposure to any stress, the WT or Δ*ecfG* strain was grown chemoheterotrophically in YPM at pH 7 and 30°C to the late stationary phase for 72 to 84 h (in 2-liter flasks with 300 ml of medium). Cells were pelleted down by centrifugation at 7,000 × *g* for 20 min at 4°C, and triplicate cultures were resuspended either in 200 ml of regular YPM or YP with the stressor and incubated overnight with shaking at 30°C in the dark. Subsequently, cells were pelleted by centrifugation at 7,000 × *g* for 20 min at 4°C and washed once with 10 ml of YPM. The cell pellets were frozen at −80°C until ready for extraction. Lipid extraction and analysis by high-temperature gas chromatography-mass spectrometry was performed similar to a previously described method (7). Cells were suspended in 2 ml of water and transferred into Teflon centrifuge tubes (VWR, Bridgeport, NJ), followed by the addition of 5 ml of methanol and 2.5 ml of dichloromethane (DCM), and sonicated for 15 min at room temperature. Samples were centrifuged at 6,000 × *g* for 10 min at 22°C, and the supernatants were transferred to new tubes. The cell pellets were sonicated again and centrifuged, and the supernatants were combined with the first extraction. The samples were separated into two phases by adding ~13 ml of DCM and centrifuged at 6,000 × *g* for 10 min at 22°C. The organic phase was transferred to a new vial and evaporated in a chemical hood overnight and further dried using a rotary evaporator under vacuum for at least 10 min. The total lipid extract (TLE) was resuspened in DCM at a concentration of 1 mg/ml. Then, 100 μl of this extract was combined with 1 μl of an internal standard (750 ng of *trans*-androsterone/μl), followed by evaporation under N_2. The TLE was then derivatized to acetate esters by incubation in 100 μl of acetic anhydride-pyridine (1:1) for 30 min at 60°C. Peak areas of hop-17(21)-ene, hop-22(29)-ene, hop-21-ene, diplopterol, BHT, and their respective 2-methylated species were integrated and compared to those from androsterone standards to obtain the yields from TLE.

RESULTS AND DISCUSSION

Alphaproteobacterial GSR regulatory components are conserved in *R. palustris*. A single EcfG ortholog (Rpal_4706) was found in *R. palustris* TIE-1 using a genome-wide BLASTP search with the *B. japonicum* EcfG sequence (protein sequence identities among orthologs are reported in Table S2 in the supplemental material). NepR (Rpal_4705) and PhyR orthologs (Rpal_4704) were similarly identified upstream of EcfG, a finding consistent with the microsynteny observed for GSR components in other alphaproteobacteria (19). Although *nepR* is in an operon with *ecfG* such that the stop codon of *nepR* overlaps with the start codon of *ecfG*, *phyR* is transcribed divergently from this operon (Fig. 2A). Multiple sequence alignment of characterized PhyRs with Rpal_4704 indicated that the phosphorylation site, aspartate 189 (D189), is conserved in this protein (see Fig. S1A in the supplemental material) (15). Finally, Rpal_4707, encoding a putative periplasmic protein was found downstream of the *nepR-ecfG* operon. Interestingly, not only does Rpal_4707 possess a Pfam: HWE-HK domain that is characteristic of some histidine kinases genetically linked to the GSR loci, it also contains the conserved histidine residue (H347) that gets phosphorylated to initiate transduction of the stress signal (see Fig. S1B in the supplemental material) (19). Notably, the genomic organization of these four regulatory proteins is conserved in all *R. palustris* strains (20).

Prior studies in alphaproteobacteria led to the identification of a consensus EcfG-binding motif, $GGAACN_{16\text{-}17}cgTT$, wherein GGAAC and cgTT comprise the −35 and −10 regions, respectively, of stress-related gene/operon promoters and N symbolizes any nucleotide base (10, 11, 14). Although the lowercase bases are not always present in the motif, the uppercase bases are well conserved; however, EcfG has been shown to tolerate one to two substitutions in them (16). To identify potential EcfG targets, we screened the *R. palustris* TIE-1 genome for the presence of the canonical EcfG motif ($GGAACN_{18\text{-}19}TT$) upstream of genes and operons (see Table S3 in the supplemental material). As in other alphaproteobacteria, the motif is present upstream of *nepR-ecfG* operon and *Rpal_4707* but absent in the *phyR* upstream region (Fig. 2B). The AAC and TT motif bases are considered to be the most important EcfG binding determinants; however, *phyR* even lacks the $AACN_{18\text{-}19}TT$ motif (16). Interestingly, within the putative hopanoid biosynthesis cluster, the consensus (or minimal) motif was only found upstream of the C-2 hopanoid methylase gene *hpnP*. In addition, two motifs with imperfect spacing were identified upstream of Rpal_4266, which encodes a hypothetical protein and the hopanoid transporter, *hpnN* (25). Although the *hpnP* EcfG motif is conserved among all *R. palustris* strains (see Fig. S2 in the supplemental material), this is not the case for either of the other hopanoid gene motifs. We further investigated the regulation of these genes by EcfG.

EcfG regulates hpnP expression under high-temperature stress in a PhyR-dependent manner. To check whether EcfG mediates a stress response in *R. palustris*, we measured expression of EcfG and its putative target genes under high-temperature stress using quantitative reverse-transcription PCR (qRT-PCR) (Fig. 2B). This involved quantifying transcript levels in log-phase cultures of the WT strain exposed to 30°C (ambient) and comparing them to cells exposed to 42°C (nonpermissive) for 30 min. We used *in vitro* gene transcripts of known concentrations as standards. Expression of both *nepR* and *ecfG* increased significantly with heat stress, which is consistent with the presence of the canonical motif upstream of the *nepR-ecfG* operon (Fig. 2B). In contrast, despite an upstream motif, *Rpal_4707* expression showed minimal upregulation (>2-fold), whereas *phyR* with no apparent motif showed considerable upregulation in expression. From the hopanoid gene cluster, we determined the expression of the three genes mentioned above and a fourth gene that does not contain an upstream EcfG-binding motif, *shc* (squalene hopene cyclase, which catalyzes the cyclization step in hopanoid biosynthesis) (7). Of all of these genes, only *hpnP* transcription levels were increased by heat stress. This is not surprising given the lack of a strong consensus motif upstream of *hpnN* or Rpal_4266. Thus, all genes within the *R. palustris* GSR regulatory locus and one gene within the hopanoid biosynthesis cluster responded to heat stress by upregulating their expression.

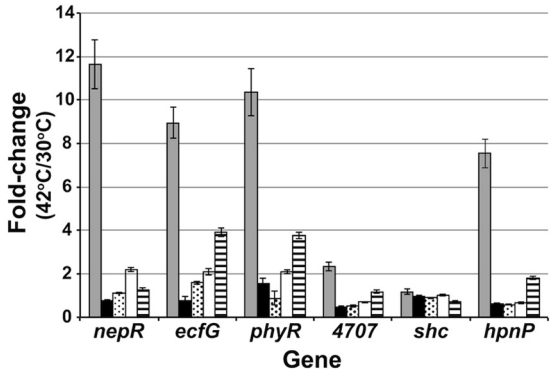


FIG 3 Expression analyses of heat shock-responsive genes in various regulator mutant backgrounds. The fold upregulation in transcription was measured under heat shock for all genes within the GSR locus, the *hpnP* gene, and the control gene *shc* using qRT-PCR in the WT (DKN379, gray), Δ*ecfG* (DKN1216, black), Δ*phyR* (DKN1219, dotted), *phyR*(D189A) (DKN1285, white), and P*lac-nepR* (DKN1251, *nepR* overexpression strain, striped) strains. Each value represents the average and standard error for three biological replicates. Upregulation was abolished in all mutants and significantly reduced in the *nepR* overexpression strain.

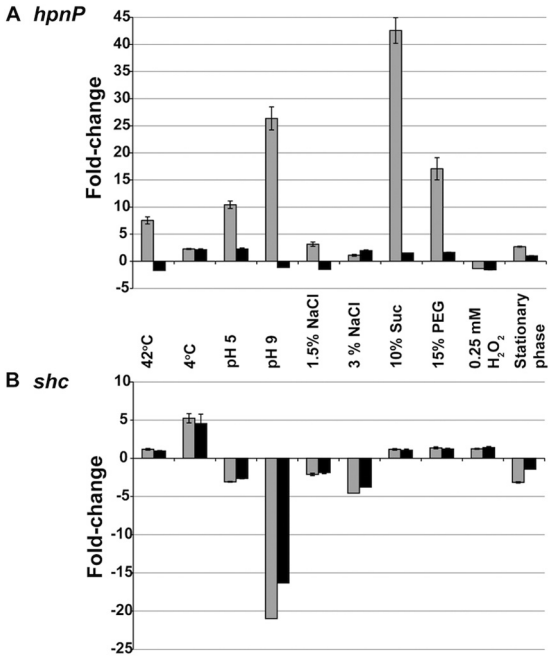


FIG 4 Expression analyses of *hpnP* (A) and *shc* (B) in WT (DKN379; gray bars) and Δ*ecfG* (DKN1216; black bars) strains under a variety of stress conditions using qRT-PCR. Each value represents the average and standard error of three biological replicates. In the WT strain, a >5-fold increase in *hpnP* transcription was observed at 42°C, pH 5, pH 9, 10% sucrose, and 15% PEG. In contrast, *shc* transcription changed >5-fold only at 4°C and pH 9. Although *hpnP* upregulation was abolished in the Δ*ecfG* mutant, *shc* expression was still regulated in this mutant.

To determine whether this response was due to the GSR regulatory components, we performed deletion analyses. Upregulation of all genes was abolished (<2-fold) in a Δ*ecfG* mutant, implying EcfG mediates their expression under stress (Fig. 3). Upregulation of *nepR*, encoded within the *nepR-ecfG* operon, by EcfG suggests that, as in other alphaproteobacteria, *ecfG* is autoregulated (9, 11, 13, 14). It is unclear whether EcfG controls stress-related expression of *phyR* directly by binding to a nonconsensus motif (the promoter region of *phyR* lacks the canonical EcfG motif) or indirectly by inducing expression of another regulator. To determine whether PhyR is required for EcfG regulation, we constructed a Δ*phyR* mutant and a site-directed *phyR*(D189A) mutant with an aspartate (D) to alanine (A) substitution of the conserved phosphorylation residue D189 (15). In both mutants, upregulation of the set of genes we tracked was abolished (Fig. 3). Because the conserved phosphorylation residue is critical for PhyR function, our results demonstrate that PhyR is necessary for expression of EcfG targets under stress in likely the same manner as in other alphaproteobacteria (15). Deletion of the anti-σ factor, *nepR*, has been shown to be deleterious in some alphaproteobacteria (11); therefore, an IPTG-inducible *nepR* overexpression strain (P*lac-nepR*) instead of a deletion strain was constructed to verify its role in the regulatory pathway. Although regulation of none of the tracked genes was completely abolished in the P*lac-nepR* strain, it was greatly reduced, corroborating the negative effect of *nepR* on expression of EcfG targets (Fig. 3). Finally, we attempted to generate a *Rpal_4707* deletion mutant but, despite screening ~200 colonies, we were never able to isolate one. As an alternative approach, we tried to only mutate the conserved catalytic H347 amino acid of Rpal_4707 to alanine (H347A), and yet again each segregant colony screened retained the WT H347 residue. This suggests that *Rpal_4707* is essential for the growth of *R. palustris* under the conditions tested. In the absence of a Δ*Rpal_4707* mutant, we were unable to directly test its involvement in the regulation of EcfG target genes under stress.

***hpnP* expression is upregulated under a variety of stresses in an EcfG-dependent manner.** As shown above, *hpnP* is part of the EcfG regulon, suggesting the 2-MeBHPs are somehow involved in dealing with stress. We were curious to find out the suite of stresses under which *hpnP* is upregulated. We monitored, in addition to *hpnP*, *shc* transcription as a control and to gauge the relative regulation of total BHP versus 2-MeBHP synthesis (Fig. 4). For each stress, we exposed log-phase cultures of WT and Δ*ecfG* strains to a growth-limiting or growth-inhibitory condition for 30 min. Subsequently, we calculated the fold regulation relative to the ambient condition. As shown above, under high-temperature stress, *hpnP* expression increased ~8-fold in an EcfG-dependent manner. In contrast, no upregulation (~2-fold in WT and Δ*ecfG* strains) was seen with a cold shock at 4°C. *shc* transcription stayed constant under high-temperature stress but increased ~5-fold at 4°C. This marginal increase in *shc* expression was seen in the Δ*ecfG* mutant as well, implying that it is not due to EcfG.

Relative to ambient pH 7, *hpnP* expression exhibited significant increases of ~10- and ~26-fold under acidic (pH 5) and alkaline (pH 9) conditions, respectively. Interestingly, although *shc* transcription did not change appreciably (~3-fold) at pH 5, it decreased ~21-fold at pH 9 in an EcfG-independent manner. Next, we tested the response of these genes to osmotic stress by ionic (sodium chloride, NaCl) and nonionic (sucrose and PEG) solutes. Growth-limiting concentrations of 1.5% NaCl (257 mM) and 3% NaCl (500 mM) did not lead to a change in *hpnP* or *shc* expression compared to the 0% NaCl control (<5-fold). However, exposure to either of the nonionic solutes, 10% sucrose (292 mM) or 15% PEG, increased *hpnP*, but not *shc* (<2-fold), transcription by 43- and 17-fold, respectively. This upregulation went

TABLE 2 Hopanoid quantification in *R. palustris* TIE-1 strains under various stress conditions[a]

Growth condition[b]	WT (μg/mg TLE)		Δ*ecfG* mutant (μg/mg TLE)		WT/Δ*ecfG* mutant[c]	
	Total BHPs, % 2-Me	Total BHT, % 2-Me	Total BHPs, % 2-Me	Total BHT, % 2-Me	Total BHPs, % 2-Me	Total BHT, % 2-Me
Temp						
30°C	66 ± 7, 30 ± 5	10 ± 2, 7 ± 2	67 ± 2, 14 ± 1	10 ± 1, 3 ± 1	0.82, 5.1E–3	0.94, 0.023
39°C	58 ± 11, 25 ± 1	6 ± 2, 7 ± 1	79 ± 11, 13 ± 1	8 ± 1, 2 ± 1	0.081, **4.4E–4**	0.16, **1.3E–3**
P	0.36, 0.22	0.089, 0.88	0.13, 0.68	0.034, 0.17		
pH 7 and 5						
pH 7	66 ± 4, 42 ± 1	13 ± 1, 10 ± 1	58 ± 1, 20 ± 1	13 ± 1, 3 ± 1	0.034, **6.9E–8**	0.87, **1.5E–5**
pH 5	70 ± 10, 43 ± 3	8 ± 3, 15 ± 2	63 ± 6, 14 ± 1	8 ± 1, 3 ± 1	0.39, **1.1E–4**	0.81, **1.7E–4**
P	0.58, 0.62	0.081, **4.2E–3**	0.26, **1.7E–4**	**7.5E–3**, 0.60		
pH 7 and 9						
pH 7	66 ± 1, 37 ± 1	13 ± 1, 7 ± 1	52 ± 1, 20 ± 1	7 ± 1, 3 ± 1	**1.6E–4**, **5.3E–6**	**1.8E–3**, **3.2E–4**
pH 9	55 ± 8, 40 ± 1	9 ± 2, 12 ± 1	55 ± 5, 15 ± 1	6 ± 1, 3 ± 1	0.96, **2.0E–6**	0.071, **3.6E–5**
P	0.065, **2.4E–3**	0.036, **6.5E–4**	0.25, **0.0010**	0.32, 0.078		
Suc						
No suc	51 ± 2, 43 ± 1	8 ± 1, 9 ± 1	64 ± 12, 12 ± 1	6 ± 1, 3 ± 1	0.14, **2.7E–7**	0.016, **2.2E–5**
10% suc	42 ± 6, 58 ± 6	7 ± 1, 16 ± 3	78 ± 9, 12 ± 1	6 ± 1, 4 ± 1	5.1E–3, **1.5E–4**	0.42, **2.7E–3**
P	0.078, 0.010	0.015, 0.022	0.16, 0.65	0.81, **2.5E–3**		
PEG						
No PEG	62 ± 3, 26 ± 1	12 ± 1, 5 ± 1	60 ± 6, 15 ± 1	11 ± 1, 3 ± 1	0.64, **1.9E–6**	0.21, **7.8E–4**
15% PEG	31 ± 1, 38 ± 1	4 ± 1, 11 ± 1	29 ± 1, 15 ± 1	5 ± 1, 4 ± 1	0.080, **5.3E–6**	0.042, **1.1E–4**
P	**7.0E–5**, **5.2E–6**	**2.6E–4**, **2.9E–5**	**1.1E–3**, 0.79	**5.4E–4**, 0.27		

[a] WT, DKN379; Δ*ecfG* mutant, DKN1216; TLE, total lipid extract. BHPs, bacteriohopanepolyol or hopanoids (these include unmethylated and methylated diplopterol, diploptene, and bacteriohopanetetrol. 2-Me, 2-methyl. Each value represents the average ± the standard deviation of three biological replicates. BHT, bacteriohopanetetrol. Each value represents the average ± the standard deviation of three biological replicates. Numbers shaded in gray represent *P* values from the Student *t* test using double-tailed distribution and two-sample equal variance analyses. *P* values of <0.005 are indicated in boldface.

[b] Each *P* value row indicates the comparison between stress and nonstress conditions. Suc, sucrose.

[c] *P* value comparisons between WT and Δ*ecfG* samples.

away completely in the Δ*ecfG* mutant, indicating EcfG was responsible for it. The different response to ionic and nonionic osmolytes is not unprecedented; for instance, in *E. coli*, 40% of genes upregulated with NaCl or sucrose treatment are unique to the osmolyte (26). The expression of both *hpnP* and *shc* remained unchanged during oxidative stress, which was imposed by exposing cultures to 250 μM hydrogen peroxide (H_2O_2). Similar results were obtained when transcription of both genes was compared between the exponential (OD_{600} of ~0.125) and stationary (OD_{600} of ~0.35) phases.

Because EcfG upregulates *hpnP* transcription under high-temperature, acidic, alkaline, and hyperosmotic conditions, it is likely that methylated hopanoids help regulate fluidity and decrease the permeability of membranes to combat these stresses. This conclusion is supported by a lack of *hpnP* induction under cold shock wherein homeoviscous adaptation is possibly achieved through incorporation of lipids that increase membrane fluidity (27). Similar scenarios can be envisioned for oxidative and stationary-phase stresses where *hpnP* transcription stayed constant. Total lipid analysis will be necessary to test these hypotheses.

2-MeBHP production increases under some stresses in an EcfG-dependent manner. To determine whether an increase in *hpnP* transcription also results in an increase in 2-MeBHPs, we quantified hopanoids in the WT strain and the Δ*ecfG* mutant under several of the aforementioned stresses and statistically analyzed their differences (Table 2). This involved measuring hopanoids in saturated cultures that had been exposed overnight to the ambient or stress condition. *R. palustris* TIE-1 produces the C_{30} hopanoids diploptene and diplopterol and the C_{35} hopanoids bacteriohopanetetrol (BHT) and aminobacteriohopanetriol. Moreover, diploptene, diplopterol, and BHT can be methylated at C-2 (Fig. 1A) (7). The total BHP contents (unmethylated and methylated diploptene, diplopterol, and BHT) of both strains did not change appreciably under any condition. Although *hpnP* transcription was upregulated under all of the stresses examined, the percentage of total 2-methyl hopanoids (2-MeBHPs) produced increased ~35 and 46% only under sucrose and PEG stress, respectively, compared to no sucrose or the PEG control. Thus, 2-MeBHP biosynthesis appears to be more responsive to osmotic stress even though *hpnP* was similarly upregulated under pH stress. Nevertheless, the percentage of 2-methyl bacteriohopanetetrol (2-MeBHT) increased by ca. 50 to 120% of total bacteriohopanetetrol under all conditions, except under high temperature. This suggests that 2-MeBHT might be the key hopanoid for dealing with stress. No increase in 2-MeBHP or 2-MeBHT content under stress was observed in the Δ*ecfG* mutant, corroborating involvement of EcfG in the overproduction of these methylated hopanoids. It is important to note that the Δ*ecfG* mutant produced fewer 2-MeBHPs, including 2-MeBHT, than the WT even in the absence of stress.

Interestingly, an increase in *hpnP* transcription was not always accompanied by a corresponding increase in 2-MeBHP produc-

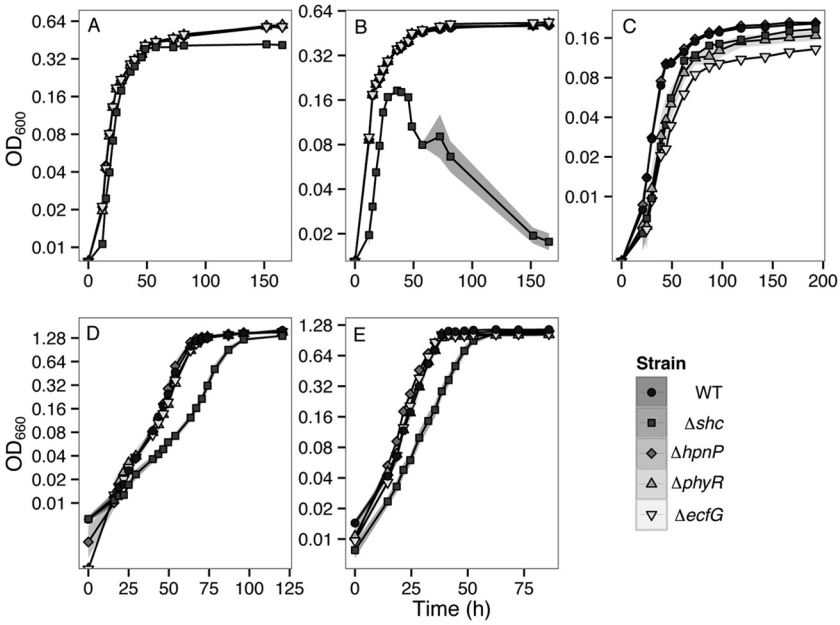


FIG 5 Growth curves of *R. palustris* strains under various physiological conditions. Growth of WT (DKN379, circle), Δ*shc* (DKN677, square), Δ*hpnP* (DKN692, diamond), Δ*phyR* (DKN1219, triangle), and Δ*ecfG* (DKN1216, inverted triangle) was monitored at an OD_{600} under chemoheterotrophic conditions at 30°C (A), 39°C (B), in the presence of 15% PEG (C), or at OD_{660} under photoautotrophic-H_2 (D) and photoheterotrophic-acetate (E) conditions. Each curve represents the average of at least three biological replicates, and the shaded area around the curve represents the standard deviation. *y* axis in log scale. Relative to the WT, the Δ*shc* mutant exhibits a growth defect under all conditions, especially at 39°C. Although the Δ*hpnP* mutant grows like the WT; the Δ*ecfG* and Δ*phyR* mutants are not able to grow as well as the WT in the presence of 15% PEG.

tion. This could be due to regulation of the *hpnP* transcript or the HpnP enzyme; *hpnP* mRNA might get turned over rapidly or translated slowly. Alternatively, HpnP enzyme activity might be affected by numerous factors such as substrate availability or the presence of other lipids. Nevertheless, 2-MeBHT levels went up slightly with each stress highlighting the importance of this C_{35} hopanoid in stress tolerance (Table 2). This result is especially relevant in light of recent work showing that C_{35} BHPs of *R. palustris* enhance the integrity of the outer membrane (8). Also, extended hopanoids have been specifically implicated in dealing with stress in other bacteria such as *Bacillus acidocaldarius* (28) and *Frateuria aurantia* (29). How HpnP discriminates between its two substrates, C_{30} versus C_{35} BHPs, and why one lipid class is more relevant under our experimental conditions remain to be determined.

2-MeBHPs are not essential for the growth of *R. palustris* under standard laboratory stress conditions. In response to stress, if 2-MeBHP production is a beneficial adaptation, then deletion of *hpnP* should have phenotypic consequences in *R. palustris*. We tested this hypothesis by monitoring the growth of the WT strain, as well as Δ*shc* (7), Δ*hpnP* (6), Δ*phyR*, and Δ*ecfG* mutants, under the stress conditions that led to upregulation of *hpnP* expression (Fig. 5). Growth at high temperature was monitored in batch cultures at 39°C instead of the nonpermissive 42°C (Fig. 5B). All mutants exhibited WT strain-like growth at this temperature, with the exception of the Δ*shc* mutant. The Δ*shc* mutant attained lower yields than the WT strain (70%) at 30°C (Fig. 5A); however, at 39°C its yields decreased to 36% that of the WT strain, followed by drastic reduction in the OD_{600}. This suggests that BHPs other than 2-MeBHPs are needed to deal with heat stress. Curiously, even the GSR regulator, EcfG, was not required under this condition. This is not uncommon; however, the Δ*ecfG* mutant of *S. meliloti* also does not exhibit any stress-related phenotypic defects (11). Notably, *R. palustris* TIE-1 encodes 17 other alternate sigma factors, 13 of which are annotated as ECF, 2 as heat shock, and 2 as σ^{54} (20, 30). These factors might regulate a unique set of stress-related genes and/or genes within the EcfG regulon, rendering it dispensable under stress. It will be interesting to identify and compare these regulons in future studies.

Growth in a range of pH values, 5 to 9, was tested by spotting 10-fold serial dilutions of each of the strains onto agar plates (Table 3; see also Fig. S3 in the supplemental material). At the

TABLE 3 Growth of *R. palustris* strains under pH and sucrose stress

Growth condition	Log_{10} of highest dilution exhibiting growth on solid medium[a]				
	WT	Δ*shc* mutant	Δ*hpnP* mutant	Δ*phyR* mutant	Δ*ecfG* mutant
pH 5.5[b]	5 (+++)	ND	5 (+)	5 (+)	5 (+)
pH 6.0	6	6	6	6	6
pH 7.0	6	6	6	6	6
pH 8.0	6	4	5	6	6
pH 8.5	4	2	4	4	4
pH 9.0	1	NG	1	1	1
No sucrose	6	6	6	6	6
10% sucrose	5	2	5	5	5
15% sucrose	2	1	2	2	2

[a] Strain numbers: WT, DKN379; Δ*shc* mutant, DKN677; Δ*hpnP* mutant, DKN692; Δ*phyR* mutant, DKN1219; Δ*ecfG* mutant, DKN216. ND, no dilution; NG, no growth.
[b] Qualitative differences in growth at pH 5 are represented in parentheses (see Fig. S3 in the supplemental material).

lowest pH that allowed the growth of the WT, pH 5.5, the Δ*shc* mutant displayed negligible growth. In contrast, the WT and the other mutants grew to a 10^{-5} dilution. Interestingly, the growth of all mutants was sparse at 10^{-2} to 10^{-4} dilutions, indicating their inability to cope with acidic conditions as well as the WT. These results could be solely attributed to low pH because all mutants were able to grow at higher pH values of 6 and 7 (control pH). This suggests that 2-MeBHPs might be important in acid tolerance. At higher pH values of 8 and 8.5, only the Δ*shc* mutant grew one to two dilutions less than the WT. Moreover, its growth at pH 9 was completely abolished, highlighting the importance of unmethylated BHPs in dealing with alkaline stress. A similar conclusion could be drawn for growth in the presence of 10 or 15% sucrose, where again the Δ*shc* mutant was the only strain to exhibit a slight growth defect. We also found EcfG to be dispensable under both alkaline and sucrose stresses.

In the presence of 15% PEG, growth of the WT in batch cultures was severely affected such that the generation time doubled and the yield was reduced by a third (Fig. 5C). Nevertheless, the Δ*hpnP* mutant grew like the WT. The most severely growth-compromised strain was the Δ*ecfG* mutant, followed by the Δ*phyR* mutant and then the Δ*shc* mutant. This finding implies that, in addition to hopanoids, cells need products of other genes within the EcfG regulon to deal with PEG stress.

Because *R. palustris* is capable of growth by photosynthesis (21), we tested the growth of all strains in the presence of light in batch cultures. Under both autotrophic (H_2 [electron donor] and CO_2 [carbon source]; Fig. 5D) and heterotrophic (acetate [electron donor and carbon source]; Fig. 5E) growth conditions, only the Δ*shc* mutant showed a slight defect. Interestingly, in the presence of acetate, but not H_2, the growth yields of the Δ*phyR* and Δ*ecfG* mutants were slightly less (8 to 11% less) than that of the WT, possibly pointing toward their role in dealing with stationary-phase stress under this condition.

In summary, we have shown that the expression of the C-2 hopanoid methylase gene *hpnP* is regulated at the molecular level by the general stress response. This provides an important clue to help us understand the physiological function of 2-MeBHPs. That the Δ*hpnP* mutant did not exhibit a strong phenotype under any of the stress conditions tested was initially surprising, and yet cells possess multiple ways to cope with a single type of stress, and these coping mechanisms are usually part of multiple regulons. In the absence of 2-MeBHPs, other lipids might compensate (31, 32). Moreover, it is possible that whereas stress is necessary to upregulate 2-MeBHP production, it is only under certain conditions that the ability to make 2-MeBHPs is essential. In light of the enrichment of *hpnP* sequences in habitats supporting plant-microbe interactions (Ricci et al., unpublished), it may be constructive to consider the environmental chemistry of the rhizosphere to better define these conditions. Going forward, we hope to identify conditions where 2-MeBHP production confers a selective benefit to the organism.

ACKNOWLEDGMENTS

This study was supported by awards from NASA (NNX12AD93G) and the Howard Hughes Medical Institute (HHMI) to DKN. DKN is an HHMI Investigator. We thank Tim Donohue for the suggestion to consider ECF sigma factor regulation of *hpnP*, Maureen Coleman for help with bioinformatic analysis and members of the Newman lab for constructive comments on the manuscript.

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