Two-Tiered Histidine Kinase Pathway Involved in Heat Shock and Salt Sensing in the General Stress Response of *Sphingomonas melonis* Fr1

Andreas Kaczmarczyk, Ramon Hochstrasser, Julia A. Vorholt, Anne Francez-Charlot

Institute of Microbiology, ETH Zurich, Zurich, Switzerland

ABSTRACT

The general stress response (GSR) allows bacteria to monitor and defend against a broad set of unrelated, adverse environmental conditions. In *Alphaproteobacteria*, the key step in GSR activation is phosphorylation of the response regulator PhyR. In *Sphingomonas melonis* Fr1, seven PhyR-activating kinases (Paks), PakA to PakG, are thought to directly phosphorylate PhyR under different stress conditions, but the nature of the activating signals remains obscure. PakF, a major sensor of NaCl and heat shock, lacks a putative sensor domain but instead harbors a single receiver (REC) domain (PakF_{REC}) N-terminal to its kinase catalytic core. Such kinases are called “hybrid response regulators” (HRRs). How HRRs are able to perceive signals in the absence of a true sensor domain has remained largely unexplored. In the present work, we show that stresses are actually sensed by another kinase, KipF (kinase of PakF), which phosphorylates PakF_{REC} and thereby activates PakF. KipF is a predicted transmembrane kinase, harboring a periplasmic CHASE3 domain flanked by two transmembrane helices in addition to its cytoplasmic kinase catalytic core. We demonstrate that KipF senses different salts through its CHASE3 domain but is not a sensor of general osmotic stress. While salt sensing depends on the CHASE3 domain, heat shock sensing does not, suggesting that these stresses are perceived by different mechanisms. In summary, our results establish a two-tiered histidine kinase pathway involved in activation of the GSR in *S. melonis* Fr1 and provide the first experimental evidence for the so far uncharacterized CHASE3 domain as a salt sensor.

IMPORTANCE

Hybrid response regulators (HRRs) represent a particular class of histidine kinases harboring an N-terminal receiver (REC) domain instead of a true sensor domain. This suggests that the actual input for HRRs may be phosphorylation of the REC domain. In the present study, we addressed this question by using the HRR PakF. Our results suggest that PakF is activated through phosphorylation of its REC domain and that this is achieved by another kinase, KipF. KipF senses heat shock and salt stress, with the latter requiring the periplasmic CHASE3 domain. This work not only suggests that HRRs work in two-tiered histidine kinase pathways but also provides the first experimental evidence for a role of the so far uncharacterized CHASE3 domain in salt sensing.

Many bacteria possess a so-called general stress response (GSR) that allows them to monitor a broad range of stressful conditions and to launch an appropriate response to adapt to adverse environments. In *Alphaproteobacteria*, the GSR is controlled by an alternative sigma factor, σ^{EcFG} , whose activity is regulated via a partner-switching mechanism (1, 2). Under nonstress conditions, σ^{EcFG} is inactivated by binding to the anti-sigma factor NepR. Upon stress, NepR is sequestered by the anti-anti-sigma factor PhyR, thereby releasing σ^{EcFG} and allowing it to activate transcription of stress-related genes. PhyR is a response regulator, and as such, its anti-anti-sigma factor activity depends on phosphorylation of a conserved Asp residue in its receiver (REC) domain. Histidine kinases belonging to the HWE (3) or HisKA₂ (4) family are emerging as stress sensors, triggering the GSR through phosphorylation of PhyR (5–10). Because HWE and HisKA₂ kinases share an HRXXN amino acid motif in their H boxes, they are collectively referred to as HRXXN kinases (7). The discovery of HRXXN kinases as PhyR kinases is relatively recent, and the actual signals that are sensed by these kinases are generally unknown.

In *Sphingomonas melonis* Fr1, seven HRXXN kinases, called PhyR-activating histidine kinases (Paks; PakA to PakG), are thought to activate the GSR by directly phosphorylating PhyR (7),

while another HRXXN kinase, PhyP, seems to be a phosphatase of PhyR, presumably preventing deleterious overactivation of the GSR under nonstress conditions (6, 11). Paks also phosphorylate the single-domain response regulator (SDRR) SdrG, which acts genetically upstream of *phyR* and plays an important role in the GSR, through a currently unknown mechanism (7). The *S. melonis* Fr1 GSR is involved in resistance to several stresses, and a number of conditions that induce the GSR in a PhyR-dependent manner have been identified (6, 7). Three of the seven Paks have been associated with particular inducing conditions, namely,

Received 9 January 2015 Accepted 3 February 2015

Accepted manuscript posted online 9 February 2015

Citation Kaczmarczyk A, Hochstrasser R, Vorholt JA, Francez-Charlot A. 2015. Two-tiered histidine kinase pathway involved in heat shock and salt sensing in the general stress response of *Sphingomonas melonis* Fr1. *J Bacteriol* 197:1466–1477. doi:10.1128/JB.00019-15.

Editor: T. J. Silhavy

Address correspondence to Anne Francez-Charlot, fanne@micro.biol.ethz.ch.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00019-15

PakB with *t*-butyl hydroperoxide (TBHP) sensing, PakC with sensing of TBHP and heat shock (HS), and PakF with HS and NaCl sensing. PakB and PakC seem to be largely redundant in TBHP sensing, i.e., either kinase alone is sufficient to almost fully activate the GSR. In contrast, PakF and PakC seem to have nonredundant roles in GSR activation by HS, since both *pakF* and *pakC* single mutants show reduced GSR activation, and this effect is additive in the corresponding double mutant (7). Although the actual signals sensed are currently unknown, PakB and PakC have classical sensory input domains (i.e., Per-Arnt-Sim [PAS] and cyclic GMP phosphodiesterase, adenylyl cyclase, FhlA [GAF] domains), suggesting that they are genuine sensor kinases. In contrast, PakF is unusual in that it does not possess a true sensor domain. Instead, it harbors a single REC domain (PakF_{REC}) N-terminal to its kinase catalytic core. Kinases with such an architecture are sometimes classified as “hybrid response regulators” (HRRs) (12), but they are largely unexplored.

It seemed plausible that the true input for PakF activation by NaCl and HS is phosphorylation of its REC domain by another kinase rather than a direct effect of these stressors on PakF. PakF is encoded in an apparent operon with the genes for the SDRR PkrF (7) and a second histidine kinase, here renamed KipF (kinase of PakF), which is a predicted transmembrane kinase with a single periplasmic CHASE3 (cyclase/histidine kinase-associated sensing extracellular 3) sensor domain (13) (Fig. 1A and B). PkrF was previously shown to be a negative regulator of the GSR, an effect that depends on PakF, and we proposed that PkrF might be a phosphate sink, competing with PhyR and SdrG for phosphorylation by PakF (7). In this study, we tested the idea that three genes—*pakF*, *pkfF*, and *kipF*—work in the same pathway and, in particular, that KipF is the actual sensor kinase of stresses, activating the GSR through PakF by phosphorylation of its REC domain.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* TOP10 (Invitrogen) was used for cloning, and *E. coli* BL21 (DE3) (Invitrogen) was used for protein overproduction. *S. melonis* Fr1 strains are described in Table 1. LB-Lennox (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used for growth of *E. coli*, and LB-Lennox or TB medium (1% tryptone) was used for growth of *S. melonis* Fr1. If not otherwise mentioned, *S. melonis* Fr1 was cultivated at 28°C. When appropriate, the following antibiotics were added to the medium: kanamycin (50 µg/ml), tetracycline (10 µg/ml), chloramphenicol (34 µg/ml), carbenicillin (50 µg/ml), and streptomycin (100 µg/ml). Inducers (IPTG [isopropyl-β-D-thiogalactopyranoside], vanillate, and cumate) were prepared as 1,000-fold stocks in water (IPTG) or ethanol (cumate and vanillate) and added at the final concentrations detailed below.

Plasmid and strain construction. Plasmids used in this study are listed in Table 1, and primers used to construct plasmids are listed in Table 2. DNA manipulations were performed according to standard protocols (14). Phusion polymerase (Thermo Scientific) was used for PCR, restriction enzymes were from Thermo Scientific, and T4 DNA ligase was from New England Biolabs.

A permanent draft of the *S. melonis* Fr1 genome is available from Integrated Microbial Genomes on the Department of Energy Joint Genome Institute website (<https://img.jgi.doe.gov/cgi-bin/w/main.cgi>). The locus tag of *kipF* is Sphme2DRAFT_1663; locus tags of other genes described in this study are listed elsewhere (7).

To construct plasmids for gene deletions in *S. melonis* Fr1, up- and downstream regions of the gene(s) to be deleted, of approximately 750 bp each, were amplified by PCR and joined using overlap PCR. The resulting fragments were cloned into pAK405 via XbaI/Acc65I. *pakF* and *pkfF* for

FIG 1 *kipF* is involved in GSR activation upon NaCl treatment and HS. (A) Schematic of the putative *kipF-pkrF-pakF* operon, drawn to scale. (B) Schematic of PakF, PkrF, and KipF protein domain architectures (not to scale). Predicted phosphorylatable His and Asp residues in kinase and REC domains, respectively, are indicated. Gray boxes in KipF symbolize predicted transmembrane segments. The dimerization and histidine phosphotransferase (DHp) and catalytic and ATP-binding (CA) domains constituting the kinase catalytic core are indicated. (C) Absolute β-Gal activities of the *nhaA2p-lacZ* fusion in the indicated genetic backgrounds, without stress (white bars) and upon stress treatment (gray bars) with the different stressors indicated. (D) RSA values calculated for the experiments in panel C. EtOH, ethanol; WT, wild type.

complementation or overexpression experiments were first cloned into ENTR plasmid pENTR4a or pENTR4b via XbaI/Acc65I and subsequently recombined into destination plasmid pVYD or pQYD by using LR Clonase II mix (Invitrogen), resulting in *syfF2-pakF* and *syfF2-pkrF* fusions. Point mutations were introduced by site-directed mutagenesis following

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description or relevant features ^a	Source or reference
<i>Sphingomonas melonis</i> strains		
JVZ857	Fr1 wild-type strain	43
JVZ1703	JVZ857 $\Delta ecfG$	15
JVZ1704	JVZ857 $\Delta phyR$	15
JVZ1953	JVZ857 $\Delta pakF$	7
JVZ2519	JVZ857 $\Delta pkrF$	7
JVZ3190	JVZ857 $\Delta kipF$	This study
JVZ3191	JVZ857 $\Delta pkrF \Delta pakF$	7
JVZ3192	JVZ857 $\Delta kipF \Delta pkrF$	This study
JVZ3193	JVZ857 $\Delta kipF \Delta pkrF \Delta pakF$	This study
Plasmids		
pAK405	Plasmid for allelic exchange and markerless gene deletions in <i>Sphingomonas</i> ; Km ^r	15
pAK405-1663	pAK405 with fusion of up- and downstream regions of <i>kipF</i> ; Km ^r	This study
pAK405-1662-3	pAK405 with fusion of up- and downstream regions of <i>kipF-pkrF</i> ; Km ^r	This study
pAK405-1661-2-3	pAK405 with fusion of up- and downstream regions of the <i>kipF-pakF</i> region; Km ^r	This study
pENTR4a	ENTR plasmid for Gateway cloning; Km ^r	7
pENTR4b	ENTR plasmid for Gateway cloning; derivative of pENTR4a encoding a GS-rich linker upstream of the MCS; Km ^r	7
pENTR4a-1661	pENTR4a with <i>pakF</i> ; Km ^r	7
pENTR4b-1661DA	pENTR4b with <i>pakF(D62A)</i> ; Km ^r	This study
pENTR4a-1661(H162N)	pENTR4a with <i>pakF(H162N)</i> ; Km ^r	This study
pENTR4a-1661REC	pENTR4a with <i>pakF_{REC}</i> (corresponding to amino acids 1 to 159); Km ^r	This study
pENTR4a-1662	pENTR4a with <i>pkrF</i> ; Km ^r	This study
pENTR4a-1662DA	pENTR4a with <i>pkrF(D66A)</i> ; Km ^r	This study
pENTR4b-1663cyt	pENTR4b with <i>kipF_{cyt}</i> (corresponding to amino acids 214 to 503); Km ^r	This study
pVH	Plasmid carrying the vanillate-inducible promoter P _{V10} and a multiple-cloning site including 3× <i>flag</i> ; Tc ^r	22
pVH-1663	pVH expressing <i>kipF-3×flag</i> under the control of P _{V10} ; Tc ^r	This study
pVH-1663(H263A)	pVH expressing <i>kipF(H263A)-3×flag</i> under the control of P _{V10} ; Tc ^r	This study
pVH-1663(RGY/AAA)	pVH expressing <i>kipF(RGY/AAA)-3×flag</i> under the control of P _{V10} ; Tc ^r	This study
pVH-1663del(CHASE3)	pVH expressing <i>kipF_{ΔCHASE3}-3×flag</i> (corresponding to a deletion of amino acids 51 to 185) under the control of P _{V10} ; Tc ^r	This study
pVYD	Destination plasmid for Gateway cloning; LR reaction results in a <i>syfp2</i> -target gene fusion expressed under the control of the vanillate-inducible promoter P _{V10} ; Tc ^r Cm ^r	22
pVY*	Empty control plasmid, obtained by LR reaction of pVYD with pENTR4a; Tc ^r	This study
pVYD-1661	pVYD with <i>pakF</i> ; Tc ^r	This study
pVYD-1661DA	pVYD with <i>pakF(D62A)</i> ; Tc ^r	This study
pVYD-1662	pVYD with <i>pkrF</i> ; Tc ^r	This study
pVYD-1662DA	pVYD with <i>pkrF(D66A)</i> ; Tc ^r	This study
pQYD	Destination plasmid for Gateway cloning; LR reaction results in a <i>syfp2</i> -“target gene” fusion expressed under the control of the cumate-inducible promoter P _{Q5} ; Tc ^r Cm ^r	11
pQYD-PakF	pQYD with <i>pakF</i> ; Tc ^r	7
pQYD-PakF(D62A)	pQYD with <i>pakF(D62A)</i> ; Tc ^r	This study
pDEST-HisMBP	Destination plasmid for Gateway cloning for IPTG-inducible overproduction of His ₆ -MBP-tagged proteins in <i>E. coli</i> ; Ap ^r Cm ^r	44
pDEST-HisMBP-1661	pDEST-HisMBP derivative for overproduction of His ₆ -MBP-PakF; Ap ^r	7
pDEST-HisMBP-1661(H162N)	pDEST-HisMBP derivative for overproduction of His ₆ -MBP-PakF(H162N); Ap ^r	This study
pDEST-HisMBP-1661REC	pDEST-HisMBP derivative for overproduction of His ₆ -MBP-PakF _{REC} ; Ap ^r	This study
pDEST-HisMBP-1663cyt	pDEST-HisMBP derivative for overproduction of His ₆ -MBP-KipF _{cyt} ; Ap ^r	This study
pET-59-DEST	Destination plasmid for Gateway cloning for IPTG-inducible overproduction of His ₆ -Trx-tagged proteins in <i>E. coli</i> ; Ap ^r Cm ^r	Novagen
pET-59-DEST-1662	pET-59-DEST derivative for overproduction of His ₆ -Trx-PkrF; Ap ^r	This study
pET-59-DEST-1662DA	pET-59-DEST derivative for overproduction of His ₆ -Trx-PkrF(D66A); Ap ^r	This study
pET26bII-PhyR	Plasmid for overproduction of C-terminally His ₆ -tagged PhyR in <i>E. coli</i> ; Km ^r	6
pET26bII-NepR	Plasmid for overproduction of C-terminally His ₆ -tagged NepR in <i>E. coli</i> ; Km ^r	6
pET26bII-SdrG	Plasmid for overproduction of C-terminally His ₆ -tagged SdrG in <i>E. coli</i> ; Km ^r	7
pAK501-nhaA2p	Reporter plasmid harboring a <i>nhaA2p-lacZ</i> transcriptional fusion; Cm ^r	7

^a Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin/carbenicillin resistance.

TABLE 2 Primers used in this study

Primer	Sequence (5' to 3') ^a	Purpose, construct
1663upF	ATTTCTAGAATGGCCGCTGGGAGCTAC	<i>kipF</i> mutant construction
1663upR	GGAAAAGGTGGCGGAAGGATCAGCACGCGGCCGAAG	<i>kipF</i> mutant construction
1663dwF	CTTCCCGCCACCTTTTCC	<i>kipF</i> mutant construction
1663dwR	ATTTGGTACCTCCCTCGTCCGATCCGGTG	<i>kipF</i> mutant construction
1662-3_up_F_new	ATTTCTAGAATCGACCTCGAAACCACCG	<i>kipF prkF</i> and <i>kipF prkF pakF</i> mutant construction
1662-3_up_R	CGACAGGAACAGGCCGAGGACGACGAAACGGTTGCG	<i>kipF prkF</i> mutant construction
1662-3_dw_F	CTCGGCCTGTTCTCTGTCG	<i>kipF prkF</i> mutant construction
1662-3_dw_R	ATTTGGTACCCTTGTCTGGTCCGCGAGGCG	<i>kipF prkF</i> mutant construction
1661-2-3_up_R	GCTTATGCCATCGCCGCGGACGACGAAACGGTTGCG	<i>kipF prkF pakF</i> mutant construction
1661-2_dw_F	CGCGCGCGCATGGCATAAGC	<i>kipF prkF pakF</i> mutant construction
1661-2_dw_R	ATTTGGTACCATAGGCGGCGATATGTCTG	<i>kipF prkF pakF</i> mutant construction
1301ENTR_R	ATTTTCTAGATTATGCCATCGCCGCGCGCAGC	Cloning of <i>pakF</i> in ENTR plasmids
1301FL_ENTR_F	ATTTGGTACCGACTGATCCTGACCCAGGTTTC	Cloning of <i>pakF</i> in ENTR plasmids
1662_ENTR_F	ATTTGGTACCGAATGAACGCCCATCAATCCG	Cloning of <i>prkF</i> in ENTR plasmids
1662_ENTR_R	ATTTCTAGAATCAGTGTTCTCTCGACCTCG	Cloning of <i>prkF</i> in ENTR plasmids
1663cytENTR_F	ATTTTGGTACCGAACCCTACTGCGCTACACG	Cloning of <i>kipF_{cyt}</i> in ENTR plasmids
1663ENTR_R	ATTTCTAGAATCATGACGCAATGTCCTG	Cloning of <i>kipF_{cyt}</i> in ENTR plasmids
1301REC_R	ATTTCTAGAATCAGGACAGCAAGGTCTCGAG	Cloning of <i>pakF_{REC}</i> in ENTR plasmids
1301REC_F	ATTTTGGTACCGACTGATCCTGACCCAGGTTTC	Cloning of <i>pakF_{REC}</i> in ENTR plasmids
1663comp_F	ATTTACTAGTGCTGGCGTAAGGCAGCGC	Cloning of <i>kipF</i> in pVH
1663comp_R	ATTTGGTACCTCTGACGCAATGTCTGACTGG	Cloning of <i>kipF</i> in pVH
1661(D62A)_F	CGATCTCGTCGCGGTCGCCATTACATGCCGGA	Site-directed mutagenesis, <i>pakF</i> (D62A)
1661(D62A)_R	TTTCCGCGCATGTAATGGCGCACCGCGACGAGATCG	Site-directed mutagenesis, <i>pakF</i> (D62A)
1662D66A_F	CTGGTGCTGCTCGCCCTCAACCTGCC	Site-directed mutagenesis, <i>prkF</i> (D66A)
1662D66A_R	GGCAGGTTGAGGGCGAGCAGCACCAG	Site-directed mutagenesis, <i>prkF</i> (D66A)
1663(RGY-AAA)_F	GAGCGAAGCCTCGCGTCGCGCCGCGCTGCTGACCGGCGACG	Site-directed mutagenesis, <i>kipF</i> (RGY/AAA)
1663(RGY-AAA)_R	CGTCGCCGTCAGCAGCGCGGCCGACGCGAGGCTTCGCTC	Site-directed mutagenesis, <i>kipF</i> (RGY/AAA)
1663(H263A)_F	CCTATATCGTCAGCGCGGACCTCGGTTTCGCC	Site-directed mutagenesis, <i>kipF</i> (H263A)
1663(H263A)_R	GGCGAAGCGAGGTCCGCGCTGACGATATAGG	Site-directed mutagenesis, <i>kipF</i> (H263A)
1661-H162N/Y/D_F	CTGTCCGAAGTGAACDACCAGCGTCGCCAATTC	Site-directed mutagenesis, <i>pakF</i> (H162N)
1661-H162N/Y/D_R	GAATTGGCGACGCGGTHGTTCACTTCGGACAG	Site-directed mutagenesis, <i>pakF</i> (H162N)
1663DEL1_F	GGCGAACAGCACACCGAACAGCGCGCCAGCGTCCGC	Deletion of CHASE3 domain in KipF, <i>kipF_{ΔCHASE3}</i>
1663DEL1_R	GCGGACGCTGGCGCGCTGTTCTGGTGTGCTGTTCCGC	Deletion of CHASE3 domain in KipF, <i>kipF_{ΔCHASE3}</i>

^a Restriction sites used for cloning are underlined. Note that primers 1661-H162N/Y/D_F and 1661-H162N/Y/D_R each contain a degenerate nucleotide, such that the mutant allele can encode either an Asn, Tyr, or Asp substitution for His162.

the QuikChange protocol (Agilent) into ENTR plasmids carrying wild-type *pakF* or *prkF*, with the exception of the *pakF*(D62A) allele, which was obtained by overlap PCR and cloning into pENTR4b via XbaI/Acc65I. *kipF* was cloned into pVH via BcuI/Acc65I, resulting in a *kipF*-3×*flag* fusion. *kipF* mutant alleles were obtained by overlap PCR and cloning into pVH via BcuI/Acc65I. *kipF_{cyt}* was cloned into pENTR4b via XbaI/Acc65I. Plasmids for protein overproduction in *E. coli* were obtained by Gateway cloning, using ENTR plasmids carrying *pakF* or *prkF* alleles or *kipF_{cyt}* as indicated (Table 1), with pDEST-HisMBP or pET-59-DEST (Invitrogen) as the destination plasmid. pDEST-HisMBP was a gift from David Waugh (Addgene plasmid 11085).

S. melonis Fr1 in-frame gene deletion mutants were obtained using pAK405 derivatives (Table 1) and a previously published method for markerless gene deletion (15). Note that Δ *kipF* Δ *prkF* and Δ *kipF* Δ *prkF* Δ *pakF* strains were constructed by deleting the two and three genes, respectively, in one step, similar to the construction of the Δ *prkF* Δ *pakF* strain described previously (7).

β-Gal assays. All strains used in β-galactosidase (β-Gal) assays harbored plasmid pAK501-nhaA2p, carrying the *nhaA2p-lacZ* transcriptional fusion; additional plasmids carried by strains are indicated in the figures or figure legends. β-Gal assays were performed according to the method of Miller (16), and measurements represent the means ± standard deviations (SD) for at least three biological replicates. For epistasis and overexpression experiments and Δ *prkF* complementation, cultures were inoculated from a single colony into 20 ml LB-Lennox medium and grown in 100-ml baffled flasks overnight to exponential phase, and β-Gal

activity was measured. For overexpression experiments, β-Gal activity was measured before and 2 and 4 h after induction with 25 μM cumate. For all stress induction experiments, strains were grown in TB medium. The reason for using TB medium in these experiments was that *nhaA2p-lacZ* activity, i.e., GSR activity, is 3- to 4-fold lower in this medium than in LB-Lennox and GSR induction upon stress treatment cannot be observed in LB-Lennox (7). Briefly, 20 ml of TB medium in a 100-ml baffled flask was inoculated with a large loop of bacteria and grown during the day (precultures); precultures were then used to inoculate 100-ml main overnight cultures in TB medium in 500-ml baffled flasks at an optical density at 600 nm (OD₆₀₀) of 0.0001 to 0.0004. The following day, cultures (OD₆₀₀ of 0.02 to 0.10) were split into 10-ml subcultures in 100-ml baffled flasks. Subcultures were either mock treated with double-distilled water (ddH₂O) (control), shifted to 37°C (HS), or treated with 100-fold stock solutions of TBHP (1.6 mM final concentration) or ethanol (1% final concentration), 20-fold stock solutions of glycerol or sucrose (100 mM final concentration), or 20-fold stock solutions of LiCl, NaCl, KCl, MgCl₂, NaNO₃, or Na₂SO₄ (50 mM final concentration). After 1 h of incubation, β-Gal activity was measured. Cultures of strains harboring plasmid-borne *pakF* and *prkF* alleles expressed from the P_{V10} promoter (parent plasmid pVYD) contained 200 μM vanillate. Cultures of strains harboring plasmid-borne *kipF* alleles expressed from the P_{V10} promoter (parent plasmid pVH) contained 25 μM vanillate. Relative stress activation (RSA) was calculated as described before (7) and as detailed in the following. For a single biological replicate, all strains shown in one figure/subfigure were grown in parallel, always including the wild type as a reference, and stress

induction and measurement of β -Gal activity were performed in parallel. For a single experiment, RSA was calculated from absolute β -Gal activity by subtracting the β -Gal activity of the unstressed cultures from the β -Gal activity of the stressed cultures and then expressed relative to this value for an otherwise isogenic wild-type strain. RSA means and SD shown in the figures were then calculated using RSA values from three such independent experiments. Note that in cases where mutants were complemented, “wild type” refers to complementation with the wild-type allele (i.e., *kipF*-3 $\times$ flag, *syfp2-pakF*, or *syfp2-pkrF*), and “mutant control” refers to complementation with an appropriate empty plasmid control (pVY* or pVH) (Table 1).

Western blots. Samples for Western blots were taken from the same cultures used for β -Gal assays; for stress induction experiments, samples were taken from the control culture after 1 h of mock treatment as described above. One-milliliter aliquots of bacterial cultures were spun down (12,000 $\times$ g, 2 min), and cell pellets were resuspended in 1 $\times$ Laemmli buffer (corresponding to an OD₆₀₀ of 10) and incubated at 99°C for 10 min. Proteins were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membranes by semidry blotting, and detected using a primary mouse anti-green fluorescent protein (anti-GFP) (GF28R; Pierce) or mouse anti-FLAG M2 (Sigma) antibody and a horseradish peroxidase (HRP)-coupled goat anti-mouse secondary antibody (Bio-Rad). As a control, the same samples were probed with rabbit anti-GroEL (*E. coli*) serum and an HRP-coupled goat anti-rabbit secondary antibody (Bio-Rad). Chemiluminescence was detected using an enhanced chemiluminescence (ECL) Western blot detection reagent (GE Healthcare).

Protein overproduction and purification. PhyR-His₆ and His₆ were overproduced (6) and purified (7) as described previously. SdrG-His₆ and His₆-MBP-PakF were overproduced and purified as described previously (7). Overproduction and purification of His₆-MBP-PakF(H162N), His₆-MBP-PakF_{REC}, His₆-MBP-KipF_{CYT}, His₆-Trx-PkrF, and His₆-Trx-PkrF(D66A) were performed in essentially the same way as that described for His₆-MBP-PakF (7). Purified proteins were stored in kinase buffer (10 mM HEPES-KOH [pH 7.9], 50 mM KCl, 10% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol [DTT]) at -20°C .

Autophosphorylation and phosphotransfer reactions. A kinase (His₆-MBP-PakF or His₆-MBP-KipF_{CYT}) was diluted to 5 μM in kinase buffer, and MgCl₂ was added to a final concentration of 10 mM. After equilibration to room temperature for 15 to 30 min, autophosphorylation was initiated by addition of 5 to 10 μCi [γ -³²P]ATP (5,000 Ci/mmol; Hartmann Analytic GmbH, Germany) per 100 μl of 5 μM kinase solution. After 5 to 10 min (for His₆-MBP-PakF) or 1.5 to 3 h (for His₆-MBP-KipF_{CYT}) of autophosphorylation, phosphotransfer reactions were initiated by mixing equal volumes of kinase solution with “response regulator mix.” The response regulator mix was made in kinase buffer supplemented with 10 mM MgCl₂ and contained the protein(s) to be tested as a substrate at 5 μM ; in the case of PhyR, the response regulator mix additionally contained NepR at a concentration of 7.5 μM . Thus, final concentrations in phosphotransfer reaction mixtures were 2.5 μM for kinases and response regulators and 3.75 μM for NepR. For phosphotransfer reactions performed at 4°C, KipF autophosphorylation was carried out at room temperature as described above; KipF~P was then chilled to 4°C for 10 min, and all following steps were performed at 4°C with precooled response regulator mix. Phosphotransfer reactions were stopped by mixing aliquots with 3 $\times$ Laemmli buffer at the time points indicated in the figures or figure legends. Samples were then subjected to 10% or 15% SDS-PAGE, gels were dried, the dye front and unincorporated ATP were removed, and gels were exposed to a phosphorimager screen. The screen was scanned using an FX Imager Pro Plus system (Bio-Rad).

Homology modeling and structure comparison. The CHASE3 domains of the *Rhodopseudomonas palustris* CGA009 histidine kinase HK9 (RPA2292) and KipF show 32.1% identity and 59.5% similarity. The structure of the KipF CHASE3 domain (amino acids 48 to 185) was modeled based on the crystal structure of the RPA2292 CHASE3 domain (Protein Data Bank [PDB] entry 3VA9), using the SWISS-MODEL workspace

(17). The QMEAN4 score of the homology model was -3.02 . The secondary structure of KipF CHASE3 was predicted based on this three-dimensional (3D) model. Structure images were generated in PyMol. To identify proteins with structural similarity to the CHASE3 domain, PDBFold (18; www.ebi.ac.uk/msd-srv/ssm) was used to perform pairwise 3D alignments, with the 3VA9 structure as the query and the whole PDB archive as the target. Structural alignment of the CHASE3 domain (PDB entry 3VA9; chain A) and the *E. coli* Tar ligand-binding domain (PDB entry 1VLT; chain B) (19) was performed in PyMol by using the “super” command on segments corresponding to residues 49 to 175 and 46 to 179, respectively, in the full-length proteins.

RESULTS

The histidine kinase KipF is involved in GSR NaCl and HS sensing. The HRR PakF is a major sensor of NaCl and HS in the GSR of *S. melonis* Fr1 (7). PakF is encoded in an apparent three-gene operon (*kipF-pkrF-pakF*) (Fig. 1A) which includes the genes for the SDRR PkrF and a predicted histidine kinase, renamed KipF, that is predicted to contain a periplasmic CHASE3 sensor domain flanked by two transmembrane helices (Fig. 1B). In order to test whether PkrF and KipF are also involved in NaCl and HS sensing, we first tested *pkrF* and *kipF* mutant strains for GSR induction by using the previously described transcriptional *nhaA2p-lacZ* fusion as a reporter of GSR activity (7). Δ *pakF* and wild-type strains carrying the *nhaA2p-lacZ* fusion were included in these experiments as controls. From absolute reporter activity, we also calculated RSA, that is, the difference in reporter activity of a particular strain between stress and nonstress conditions relative to this difference in the wild type, as described previously (7, 20, 21). The Δ *kipF* strain showed a basal level of *nhaA2p-lacZ* activity that was comparable to those of the wild type and the *pakF* mutant under nonstress conditions (Fig. 1C). The *kipF* mutant, like the *pakF* mutant, did not respond to NaCl stress and showed reduced GSR activation upon HS compared to the wild type, and it had wild-type-like responses to TBHP and ethanol treatments (Fig. 1C and D). The *pkrF* mutant displayed increased basal activity under nonstress conditions (Fig. 1C), had reduced RSA upon HS, and showed a tendency toward less RSA in response to NaCl stress, while GSR activation was normal in response to TBHP stress and increased in response to ethanol stress (Fig. 1D). These results confirm our earlier findings suggesting that PkrF is a negative regulator of the GSR (7), since its absence leads to GSR activation under nonstress conditions. Moreover, our results demonstrate that a *kipF* mutant is impaired in GSR activation in response to NaCl stress and HS but not TBHP or ethanol stress, essentially phenocopying a *pakF* mutant.

***kipF*, *pakF*, and *pkrF* act in the same genetic pathway.** As described in the introduction, the effect of *pkrF* deletion on the GSR was previously shown to depend on *pakF*, since a *pkrF pakF* double mutant showed wild-type levels of GSR activity, in contrast to the elevated GSR activity of the *pkrF* single mutant, suggesting that *pakF* and *pkrF* act in the same genetic pathway (7) (Fig. 2A). We sought to test whether *kipF* and *pakF* also act in the same genetic pathway, i.e., if PakF activity requires KipF or *vice versa*. If this is true, then one would expect a similar dependency of the Δ *pkrF* phenotype on *kipF*. Indeed, the *kipF pkrF* double mutant displayed wild-type levels of GSR activity, similar to the *pkrF pakF* double mutant (Fig. 2A), indicating that deletion of either kinase is sufficient to abrogate the effect of *pkrF* deletion. Deletion of both *pakF* and *kipF* in the *pkrF* background (Δ *kipF* Δ *pkrF* Δ *pakF* strain) showed no additive effect on GSR activity compared to that

FIG 2 PakF and PkrF activities are controlled by phosphorylation *in vivo*. (A) β -Gal activities of the *nhaA2p-lacZ* fusion in the indicated genetic backgrounds. (B) β -Gal activities of *nhaA2p-lacZ* in a *pkf* mutant expressing the indicated *pkf* alleles (plasmids pVY*, pVYD-1662, and pVYD-1662DA) (upper panel), with Western blots of the same strains (lower panels). (C) β -Gal activities of *nhaA2p-lacZ* in the indicated genetic backgrounds expressing the indicated *pkf* alleles (plasmids pVY*, pVYD-1661, and pVYD-1661DA) (upper panel), with Western blots of the same strains (lower panels). (D) β -Gal activities of *nhaA2p-lacZ* in a *pkf* mutant expressing the indicated *pkf* alleles (plasmids pVY*, pVYD-1661, and pVYD-1661DA), without stress (control) and upon addition of 50 mM NaCl (upper panel), with Western blots of the same strains (lower panels). (E) β -Gal activities of *nhaA2p-lacZ* in different genetic backgrounds upon overexpression (++) of wild-type *pkf* or *pkf*(D62A) from the cumate-inducible promoter P_{Q5} [plasmids pQYD-PakF and pQYD-PakF(D62A)] by use of 25 μ M cumate. Note that basal *nhaA2p-lacZ* activity differed depending on the medium that strains were grown in, i.e., LB-Lennox in panels A to C and E and TB medium in panel D. For details, see Materials and Methods.

in the *pkf* *pkf* or *kipF* *pkf* double mutant (Fig. 2A), further suggesting that *pkf* and *kipF* do not act in parallel but rather function in a linear genetic pathway.

Asp66 of PkrF is required for its activity *in vivo*. Based on the above-described experiments, we hypothesized that KipF might phosphorylate and thereby activate PakF, while PkrF might be phosphorylated by either PakF or KipF, with PkrF~P negatively affecting the GSR. As an initial approach to explore this model, we tested the importance of the predicted phosphorylatable Asp residues in PakF_{REC} (Asp62) and PkrF (Asp66). For PkrF, either wild-type PkrF or a PkrF(D66A) version under the control of the vanilate-inducible promoter P_{V10} on plasmid pVYD was expressed in the *pkf* mutant (22). To monitor protein levels by Western blotting, the proteins were expressed as C-terminal fusions to SYFP2, a GFP derivative. While both proteins were present at similar levels according to Western blotting, only the wild-type allele could restore wild-type levels of *nhaA2p-lacZ* activity, suggesting that the effect of PkrF on GSR activity depends on its phosphorylation (Fig. 2B). Because PkrF could simply act as a phosphate sink for PakF or KipF, rather than PkrF~P carrying out a particular effector function, we next sought to distinguish between these possibilities by replacing Asp66 with Glu. Asp-to-Glu substitutions in some cases can mimic Asp~P in response regulators (e.g., see reference 23), whereas in other cases they do not (e.g., see reference 7). If the Glu substitution rendered the protein active, this would indicate that the protein does not simply function as a phosphoshuttle, e.g., a phosphate sink. PkrF with the D66E substitution, however, failed to complement the *pkf* mutant phenotype, similar to PkrF(D66A) (data not shown). Since it is not known whether the D66E substitution really mimics the phosphorylated state in PkrF, no conclusion can be drawn from this experiment.

PakF activity is regulated by phosphorylation of its N-terminal REC domain and by KipF *in vivo*. We next tested the importance of Asp62 in PakF_{REC}. To this end, either wild-type PakF or a PakF mutant version in which Asp62 in PakF_{REC} was replaced with alanine [PakF(D62A)] were expressed under the control of P_{V10} from plasmid pVYD as C-terminal fusions to SYFP2 in the *pkf* *pkf* double mutant. Expression of wild-type PakF led to increased *nhaA2p-lacZ* activity, i.e., restored the *pkf* mutant phenotype (Fig. 2C), indicating that the fusion protein was functional. In contrast, Δ *pkf* Δ *pkf* strains carrying an empty plasmid control or expressing PakF(D62A) failed to restore the *pkf* mutant phenotype (Fig. 2C), suggesting that PakF(D62A) is non-functional under the conditions tested. Western blotting confirmed that the PakF(D62A) fusion was expressed at an even higher level than that of wild-type PakF (Fig. 2C). These results suggest that phosphorylation of Asp62 in the REC domain is required for PakF activity. Both the wild-type and D62A versions of PakF did not restore the Δ *pkf* phenotype in a *kipF* *pkf* *pkf* triple mutant (Fig. 2C), as expected from results of epistasis experiments with the *pkf* *kipF* double mutant (Fig. 2A), suggesting that PakF phosphorylation requires KipF.

To corroborate the idea that PakF_{REC} phosphorylation is important for PakF function, we next monitored GSR induction by NaCl treatment in a *pkf* single mutant expressing the above-described *pkf* alleles *in trans*. In these experiments, only the strain expressing wild-type PakF, not that expressing PakF(D62A), was able to induce the GSR when subjected to NaCl stress (Fig. 2D). For the two alleles, GSR activities under nonstress conditions and protein levels were

FIG 3 *In vitro* phosphotransfer reactions with KipF and PakF. (A) Phosphotransfer reactions (30 s) from PakF (top) and KipF (bottom) to PhyR, SdrG, PkrF, PkrF(D66A), and PakF_{REC}, as indicated. The asterisk indicates an impurity present in the KipF preparation (see the text for details). (B) Time course of phosphotransfer from KipF~P to PakF_{REC}. (C) Time course of phosphotransfer from KipF~P to PakF(H162N). The approximate position at which the PakF(H162N)~P band is expected to appear, if stably phosphorylated, is indicated to the left. RT, room temperature. (D) Time course of phosphotransfer from KipF~P to PkrF or PkrF(D66A), as indicated. The asterisk indicates an impurity present in the KipF preparation.

similar (Fig. 2D), thus supporting the idea that phosphorylation of Asp62 is important for PakF function.

We next wondered whether intramolecular phosphotransfer from Asp62 in PakF_{REC} to the predicted phosphorylatable histidine (His162) in the PakF transmitter module might be essential for PakF function, similar to His-Asp phosphoryl group shuttling in a hybrid histidine kinase (24). Overexpression of PakF from the strong, cumate-inducible promoter P_{Q5} was previously shown to activate the GSR, bypassing the need for an activating stimulus for the kinase, and the effect of PakF overexpression on GSR activity strictly depended on His162 (7). We thus reasoned that if Asp62 was truly essential for PakF activity because it served as a phosphodonor for His162, then overexpression of PakF(D62A) would fail to induce the GSR similarly to that of PakF(H162A) (7). However, PakF(D62A) overexpression did induce the GSR in both a wild-type and a $\Delta pakF$ background, albeit less than wild-type PakF overexpression did (Fig. 2E). These results suggest that phosphorylation of PakF_{REC} plays a regulatory rather than a catalytic role in PakF activity. To finally establish the epistatic relationship between *pakF* and *kipF*, we performed similar PakF overexpression experiments in a $\Delta kipF$ background. In this case, both wild-type PakF and PakF(D62A) overexpression led to activation of the GSR (Fig. 2E), indicating that PakF acts downstream of KipF. Notably, GSR activation by overexpression of wild-type PakF in the *kipF* background was reduced compared to that for the wild-type background and was similar to that with PakF(D62A) overexpression in all genetic backgrounds tested. This further supported the idea that full activation of PakF requires both KipF and Asp62 of PakF.

In conclusion, these data are in line with a model in which KipF controls PakF activity through phosphorylation of Asp62 in PakF's N-terminal REC domain. Furthermore, they conform to

the idea that the negative effect of PkrF on the GSR depends on phosphorylation of Asp66 by either PakF or KipF.

KipF phosphorylates PakF and PkrF *in vitro*. In order to directly test the hypothesis that KipF phosphorylates PakF_{REC} and that PkrF is phosphorylated by either PakF or KipF, we attempted to reconstitute these phosphotransfer reactions with purified proteins *in vitro*. Full-length PakF, PakF_{REC}, and the cytoplasmic catalytic core of KipF were purified as C-terminal fusions to His₆-MBP, and PkrF and PkrF(D66A) were purified as C-terminal fusions to His₆-Trx. C-terminally His₆-tagged PhyR, NepR, and SdrG were purified as described previously (6, 7) and served as controls, since PhyR and SdrG are known substrates of PakF (7) but are not expected to be substrates of KipF according to the results described above. Note that efficient PhyR phosphorylation requires NepR *in vitro* (7); thus, NepR was included in phosphotransfer reaction mixtures containing PhyR.

PakF phosphorylated both SdrG alone and PhyR in the presence of NepR within 30 s (Fig. 3A, upper panel), consistent with our previous report showing that PakF is a genuine and specific kinase of these two response regulators (7). PakF did not phosphorylate PkrF (Fig. 3A, upper panel), suggesting that the negative effect of PkrF on the GSR is in fact not through competition with PhyR and SdrG for phosphorylation by PakF.

KipF did not phosphorylate SdrG alone or PhyR in the presence of NepR (Fig. 3A, lower panel), congruent with epistasis experiments indicating that KipF is upstream of PakF and thus unlikely to be a direct phosphodonor for these response regulators. In contrast, KipF efficiently phosphorylated PakF_{REC}, as seen by depletion of the radiolabel from the KipF~P band (Fig. 3A, lower panel). The fact that a PakF_{REC}~P band was not observed suggested that rapid dephosphorylation occurred. In order to directly observe PakF_{REC}~P, we attempted to decrease the dephosphory-

lation rate by performing phosphotransfer reactions at 4°C instead of room temperature and following phosphotransfer over time. Indeed, the PakF_{REC}~P band was readily observed under these conditions, within as little as 10 s after the reaction was started (Fig. 3B). We next sought to test phosphotransfer from KipF to PakF_{REC} in the context of full-length PakF. Because PakF itself is a kinase and efficiently autophosphorylates within seconds to minutes, it was necessary to use a version of PakF that cannot autophosphorylate, i.e., a His₆-MBP-PakF(H162N) fusion. In phosphotransfer reaction mixtures under standard conditions (i.e., at room temperature), the KipF~P radiolabel was rapidly depleted when KipF was incubated with PakF(H162N); again, no radiolabeled band corresponding to PakF(H162N)~P was observed (Fig. 3C). We next attempted to observe PakF(H162N)~P by performing time course experiments at 4°C, but no PakF(H162N)~P band was apparent (data not shown). These results suggested that KipF efficiently phosphorylated full-length PakF and that PakF_{REC} also underwent rapid dephosphorylation in the context of the full-length protein. Overall, these experiments established KipF as a kinase of PakF.

We next tested whether KipF was able to phosphorylate PkrF. When KipF~P was incubated with PkrF, a slight depletion of the radiolabel from the KipF~P band was observed within the 30-s period that the reaction was allowed to proceed. This coincided with an accumulation of radiolabel on an unidentified band representing an impurity in the kinase preparation (marked by an asterisk in Fig. 3A, lower panel) and on a lower-molecular-weight band likely corresponding to PkrF. To gain a better resolution of the phosphotransfer reaction, we next performed time course experiments using the wild-type PkrF and PkrF(D66A) proteins, the latter of which is not expected to be phosphorylated. As shown in Fig. 3D, the KipF~P radiolabel was almost completely depleted over the course of the experiment when KipF was incubated with wild-type PkrF, but it remained constant when KipF was incubated with PkrF(D66A). Only in the reaction mixture containing wild-type PkrF did the lower-molecular-weight band, representing PkrF~P, and the “impurity” band accumulate the radiolabel. We concluded that in these *in vitro* experiments, PkrF was specifically phosphorylated by KipF on Asp66 and the phosphoryl group was subsequently transferred to a contaminating *E. coli* protein present in the kinase preparation, the identity of which is currently not known.

In summary, our data demonstrate that KipF phosphorylates both the REC domain of PakF and the SDRR PkrF but not PhyR or SdrG. Together with the *in vivo* results described above, these results suggest that activation of the GSR in response to NaCl and HS initiates with autophosphorylation of KipF, which then phosphorylates both PkrF and PakF. Active PakF, i.e., PakF phosphorylated at Asp62 in its REC domain, then phosphorylates PhyR and SdrG, triggering the partner switch that leads to GSR activation. Our results also indicate that PakF is a much better substrate for KipF than PkrF in competition experiments *in vitro* (Fig. 3A, lower panel), raising doubts about the ability of PkrF to act as an efficient phosphate sink for KipF *in vivo*.

KipF salt and heat shock sensing can be genetically uncoupled through mutations in its CHASE3 domain. Our results implicate the histidine kinase KipF as the actual sensor kinase for NaCl and HS sensing in the GSR. As mentioned above, KipF is a transmembrane kinase with a short N-terminal cytoplasmic segment followed by a transmembrane helix, a periplasmic CHASE3

domain, a second transmembrane helix, and the cytoplasmic histidine kinase module (Fig. 1B and 4A). The periplasmic CHASE3 domain is expected to be the sensor domain of KipF. The CHASE3 sensor domain is defined by a short, consecutive amino acid signature motif (Arg-Gly-Tyr/Phe, immediately followed by two aliphatic residues and an alcohol residue) (13) predicted to be at the tip of a four-helix bundle (Fig. 4A and B). In light of the lack of any functional information on this domain, we decided to characterize the role of the KipF CHASE3 domain and the importance of the conserved Arg-Gly-Tyr/Phe motif (referred to here as the RGY motif of KipF) in salt and HS sensing in *S. melonis* Fr1.

To do so, we tested the capacity of a *kipF* mutant expressing wild-type and mutant versions of KipF *in trans* to respond to NaCl stress and HS by using the *nhaA2p-lacZ* reporter. The KipF versions tested were as follows: wild-type KipF; KipF(H263A), which has the predicted phosphorylatable His residue replaced by Ala; KipF(RGY/AAA), which has the RGY signature motif amino acids replaced by Ala residues; and KipF_{ΔCHASE3}, which lacks essentially the entire periplasmic CHASE3 domain. Note that all versions carried a C-terminal 3×FLAG tag in order to follow protein levels. As expected, the strain expressing wild-type KipF responded to both NaCl stress and HS, whereas the strain expressing KipF(H263A) failed to respond to NaCl stress and showed reduced responsiveness to HS, similar to the empty plasmid control strain (Fig. 4C and D). Strains expressing KipF(RGY/AAA) or KipF_{ΔCHASE3} completely failed to respond to NaCl stress (Fig. 4C and D), suggesting that the CHASE3 domain and the RGY signature motif within are critical for this sensory function. Surprisingly, however, the same strains still responded normally to HS (Fig. 4C and D). Since all KipF versions were expressed at similar levels (or even at lower levels than that of wild-type KipF, in the case of KipF_{ΔCHASE3}) as assessed by Western blotting (Fig. 4E), this indicated that HS is sensed by KipF independently of its CHASE3 domain.

In conclusion, these findings demonstrate that NaCl and HS sensing can be genetically uncoupled, suggesting that these two conditions are sensed through distinct mechanisms. While NaCl sensing relies on the periplasmic CHASE3 domain, HS sensing must depend on some other part of KipF, possibly the transmembrane segments or the cytoplasmic kinase catalytic core itself.

KipF senses salt rather than general osmotic stress through its CHASE3 domain. Having identified the CHASE3 domain of KipF as the sensor of NaCl stress in the GSR, we next sought to test whether KipF was a general osmotic stress sensor, such as, for example, the histidine kinase EnvZ in *E. coli* (25–28). To this end, a *ΔkipF* strain expressing wild-type KipF-3×FLAG *in trans* and harboring the *nhaA2p-lacZ* reporter described above was tested for GSR activation by exposure to different ionic and nonionic osmolytes. For nonionic osmotic stress, cultures were treated with 100 mM glycerol or sucrose, which quantitatively corresponds to the same change in osmolarity as treatment with 50 mM NaCl (100 mosmol). Neither sucrose nor glycerol treatment induced the GSR (Fig. 5A), indicating that KipF does not simply sense an increase in osmolarity. When cultures were treated with different salts at 50 mM, all salts tested induced the GSR (Fig. 5A), and this response was dependent on KipF (Fig. 5B). The response was not attributable to a single ion species, since, for instance, treatments with KCl and NaNO₃, which do not share common ions, both induced the GSR to comparable levels. The magnitude of the response also did not correlate with the ionic strength of the inducer,

FIG 4 KipF senses NaCl but not HS through its CHASE3 domain. (A) Amino acid sequence of the N-terminal part of KipF. Transmembrane helices are highlighted in gray, the CHASE3 domain is indicated by a dotted line below the sequence, and the beginning of the DHp domain is indicated by a dashed line below the sequence. Domain predictions were made with SMART (42). The predicted secondary structure of the KipF CHASE3 domain (see Materials and Methods) is indicated by solid lines above the sequence. The RGY motif at the end of predicted helix 1 in the CHASE3 domain is highlighted in dark gray. The phosphorylatable His residue (His267) of KipF is indicated by an asterisk. The arrows delimit the region deleted in KipF_{ΔCHASE3}. (B) Homology model of the KipF CHASE3 domain structure (see Materials and Methods). The RGY motif is highlighted in dark gray and indicated by an arrow. Connections of helices 1 and 5 to transmembrane segments are shown schematically. (C) Absolute β-Gal activities of *nhaA2p-lacZ* in a *kipF* mutant expressing the indicated *kipF* alleles [plasmids pVH, pVH-1663, pVH-1663(H263A), pVH-1663(RGY/AAA), and pVH-1663del(CHASE3)], without stress (control), stressed with 50 mM NaCl, or shifted to 37°C (HS). (D) RSA values calculated for the experiments shown in panel C. Note that the error bars for RSA are smaller than the error bars for absolute values shown in panel C, because RSA was calculated for individual biological replicates rather than from the means and SD shown in panel C. For details, see Materials and Methods. (E) Western blots of the same strains as those used for panel C.

because Na₂SO₄ treatment induced the GSR much less than MgCl₂ treatment did, although both salt treatments result in identical increases in ionic strength. We finally tested whether induction of the GSR by the different salts depended on the RGY signature motif of the CHASE3 domain, repeating the experiments with an otherwise isogenic strain harboring KipF(RGY/AAA) instead of wild-type KipF (Fig. 5B). Treatment with most salts failed to induce the GSR; the one exception was LiCl treatment, which was still able to induce the GSR.

In summary, our results suggest that KipF is a sensor of salt stress rather than a sensor of general osmotic stress. KipF seems to sense most of these salts through its CHASE3 domain and the RGY signature motif defining CHASE3 domains. Nevertheless, the fact that LiCl stress can still be sensed in the absence of the RGY motif suggests that not all salts are sensed in exactly the same way.

DISCUSSION

In the present work, we established that the kinase PakF works together with another histidine kinase, KipF, in a two-tiered histidine kinase pathway. According to the current model (Fig. 6A), KipF is the sensor of salt stress and heat shock and, upon autophosphorylation, transfers the phosphoryl group to the REC domain of PakF, thereby activating PakF. PakF then phosphorylates PhyR and SdrG, which, in turn, trigger the partner switch inducing the GSR. KipF also phosphorylates PkrF, which somehow restricts KipF/PakF-dependent GSR activation.

Conservation of *kipF-pkrF-pakF* in Alphaproteobacteria. PhyR is conserved throughout essentially all free-living members of the Alphaproteobacteria (29, 30), and the *kipF-pkrF-pakF*

operon is conserved in a subset of alphaproteobacterial species, notably in many members of the orders *Sphingomonadales* and *Rhizobiales* (12) (Fig. 6B). It seems likely that in those organisms, the three proteins work together in a similar way as in *S. melonis* Fr1, ultimately activating the GSR through PhyR phosphorylation. Some *Agrobacterium* and *Rhizobium* species contain two HRRs with a domain architecture similar to that of PakF, i.e., they harbor a REC domain followed by an HRXXN-type kinase catalytic core (12). In these cases, one HRR is usually encoded in a *kipF-pkrF-pakF*-like operon, whereas the second HRR is encoded in a similar operon, but with the second kinase harboring a phytochrome input domain, which possibly responds to red/far-red light (31). Thus, two KipF-PakF-like pathways that respond to different conditions might regulate the GSR in these bacteria. In yet other organisms, such as *Brucella* spp., no HRR of the HRXXN type is found, but an HRXXN kinase with a single CHASE3 domain is present (12), suggesting that CHASE3-dependent regulation of the GSR occurs through a simpler mechanism. Indeed, in *Brucella abortus*, this protein (Bab1_1673) was proposed to be involved in GSR regulation (8). It is currently not clear why a two-tiered histidine kinase pathway should be advantageous over a prototypical two-component system with a single kinase. Similar to His-Asp phosphorelays (24), such a multilayered signal transduction pathway may offer more levels of regulation, e.g., through multiple sensor kinases that converge on the HRR or through interconnections to other signal transduction pathways through the SDRR or the upstream kinase.

Roles of PakF_{REC} and PkrF phosphorylation. While our re-

FIG 5 KipF is a general salt sensor rather than a general osmotic stress sensor. (A) β -Gal activities of *nhaA2p-lacZ* in a *kipF* mutant complemented with plasmid-borne wild-type *kipF* (plasmid pVH-1663) upon treatment with different osmolytes, as indicated. (B) β -Gal activities of *nhaA2p-lacZ* in a *kipF* mutant carrying an empty plasmid control (EV; plasmid pVH) or expressing the plasmid-borne *kipF*(RGY/AAA) allele [RGY/AAA; plasmid pVH-1663(RGY/AAA)] upon treatment with different salts, as indicated.

sults show that phosphorylation of PakF_{REC} is required for its activity *in vivo*, the underlying molecular mechanism of this phosphorylation-dependent activation is currently unclear. HRRs are generally not well described, and to our knowledge, only one HRR

that has a domain architecture similar to that of PakF (a single REC domain followed by the kinase catalytic core) has been studied, namely, ExsG from *Rhizobium* NT-26 (32). For ExsG, it was shown that phosphorylation of the REC domain altered the ExsG conformation and decreased ExsG autokinase activity *in vitro*, which led the authors of that study to suggest that the phosphorylated REC domain inhibits kinase core activity (32). A conceptually similar mechanism has been proposed for the HRR CheS₃ from *Rhodospirillum centenum* (33), which harbors two REC domains and a PAS domain N-terminal to the kinase catalytic core, such that phosphorylation of one of the REC domains stimulates autodephosphorylation of the kinase catalytic core (34). Thus, the net outcome of REC domain phosphorylation in both ExsG and CheS₃ is inhibition of kinase activity. Although phosphorylation of PakF_{REC} activates rather than inhibits PakF activity, one can imagine a similar mechanism, for example, the phosphorylated REC domain may stimulate PakF kinase activity. However, preliminary *in vitro* experiments testing His₆-MBP-PakF and His₆-MBP-PakF(D62A) autophosphorylation in the presence or absence of the small phosphodonor acetyl phosphate failed to support such a model (data not shown). Clearly, future studies will be required to uncover the mechanistic details of PakF activation by REC domain phosphorylation.

The mechanism by which PkrF affects the KipF-PakF pathway is also not clear. Our results from *in vitro* phosphotransfer reactions show a clear preference of KipF for PakF over PkrF and suggest that PkrF displays rather slow dephosphorylation, somewhat arguing against a role of PkrF as a dedicated phosphate sink. However, this might be an artifact of the fusion constructs used in these experiments. We also cannot exclude the possibility that, *in vivo*, PkrF phosphorylation and dephosphorylation rates are modulated by accessory proteins or other factors or that PkrF protein levels are substantially higher than PakF protein levels, hence making PkrF an efficient phosphate sink. Unless some *pkrF* allele can be identified that uncouples PkrF phosphorylation from PkrF activity, it is difficult to exclude a role of PkrF as a phosphate sink for KipF.

FIG 6 Model of GSR regulation by the KipF-PakF pathway and conservation in other *Alphaproteobacteria*. (A) Simplified model of GSR regulation by the KipF-PakF histidine kinase pathway. Arrows indicate activating relationships, and bars represent inhibitory relationships. The dotted arrow indicates a likely indirect effect of SdrG on PhyR. Note that other HRXXN kinases (Paks) that activate the GSR through PhyR and SdrG phosphorylation and the PhyR phosphatase PhyP were omitted from the model for visual clarity. (B) Conservation of the *kipF-pkrF-pakF* operon in other *Alphaproteobacteria*. *pakF* orthologs are highlighted in dark gray, and their locus tags are given as a reference. Sensor domain annotations are according to the MiST2 database (13).

Stress sensing by KipF and CHASE3 domains. Our results indicate that KipF senses salt stress but not general osmotic stress through its CHASE3 domain, whereas HS sensing does not depend on this domain. It is thus likely that either the transmembrane segments or the kinase catalytic core itself is responsible for HS sensing, perhaps by perceiving changes in some membrane properties, as proposed for the thermosensing histidine kinase DesK of *Bacillus subtilis* (35, 36). The second sensory function of KipF, which requires the CHASE3 domain, seems to relate to salts in general rather than to a general effect of osmotic upshift or the sensing of a particular ion species (Fig. 5). It is currently not clear whether there is a common activating stimulus following salt treatment or whether ion species are sensed individually. However, the fact that LiCl sensing, in contrast to sensing of all other salts tested, did not entirely depend on the RGY motif in the CHASE3 domain of KipF (Fig. 5C) suggests that, somehow, not all salts are sensed by exactly the same mechanism.

The structure of the RPA2292 CHASE3 domain aligns best to the structures of the Tsr (PDB entry 3ATP) (37) and Tar (PDB entry 1VLT) (19) chemoreceptor sensor domains. Interestingly, the aspartate- and serine-binding pockets in Tar and Tsr are located at the tip of the four-helix bundle (19, 37), and the equivalent region in the CHASE3 domain harbors the conserved RGY motif. Several residues (Arg64, Ser68, Arg69, and Arg73 in Tar) that are involved in substrate binding in Tar and Tsr (19, 37–41) superimpose with residues within and around the RGY motif (Glu67, Arg71, Gly72, and Thr76 in RPA2292). These residues are conserved not only in the CHASE3 domain of KipF but in many CHASE3 domains in general, and Glu67 and Arg71, as charged residues, are potentially well suited to sense ions through electrostatic interactions. It is thus tempting to speculate that CHASE3 stimulus perception occurs in this region, maybe through a direct interaction with ions. Clearly, further studies will be needed to test these ideas.

ACKNOWLEDGMENTS

We thank Benjamin Gourion for comments on the manuscript.

This work was supported by Swiss National Science Foundation (SNF) grant 310030B-152835.

REFERENCES

1. Francez-Charlot A, Frunzke J, Reichen C, Ebner JZ, Gourion B, Vorholt JA. 2009. Sigma factor mimicry involved in regulation of general stress response. *Proc Natl Acad Sci U S A* 106:3467–3472. <http://dx.doi.org/10.1073/pnas.0810291106>.
2. Francez-Charlot A, Kaczmarczyk A, Fischer HM, Vorholt JA. 9 January 2015. The general stress response in Alphaproteobacteria. *Trends Microbiol* <http://dx.doi.org/10.1016/j.tim.2014.12.006>.
3. Karniol B, Vierstra RD. 2004. The HWE histidine kinases, a new family of bacterial two-component sensor kinases with potentially diverse roles in environmental signaling. *J Bacteriol* 186:445–453. <http://dx.doi.org/10.1128/JB.186.2.445-453.2004>.
4. Finn RD, Bateman A, Clements J, Coghill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer EL, Tate J, Punta M. 2014. Pfam: the protein families database. *Nucleic Acids Res* 42:D222–D230. <http://dx.doi.org/10.1093/nar/gkt1223>.
5. Foreman R, Fiebig A, Crosson S. 2012. The LovK-LovR two-component system is a regulator of the general stress pathway in *Caulobacter crescentus*. *J Bacteriol* 194:3038–3049. <http://dx.doi.org/10.1128/JB.00182-12>.
6. Kaczmarczyk A, Campagne S, Danza F, Metzger LC, Vorholt JA, Francez-Charlot A. 2011. Role of *Sphingomonas* sp. strain Fr1 PhyR-NepR-^{EcFG} cascade in general stress response and identification of a negative regulator of PhyR. *J Bacteriol* 193:6629–6638. <http://dx.doi.org/10.1128/JB.06006-11>.
7. Kaczmarczyk A, Hochstrasser R, Vorholt JA, Francez-Charlot A. 2014. Complex two-component signaling regulates the general stress response in Alphaproteobacteria. *Proc Natl Acad Sci U S A* 111:E5196–E5204. <http://dx.doi.org/10.1073/pnas.1410095111>.
8. Kim HS, Willett JW, Jain-Gupta N, Fiebig A, Crosson S. 2014. The *Brucella abortus* virulence regulator, LovHK, is a sensor kinase in the general stress response signalling pathway. *Mol Microbiol* 94:913–925. <http://dx.doi.org/10.1111/mmi.12809>.
9. Lourenco RF, Kohler C, Gomes SL. 2011. A two-component system, an anti-sigma factor and two paralogous ECF sigma factors are involved in the control of general stress response in *Caulobacter crescentus*. *Mol Microbiol* 80:1598–1612. <http://dx.doi.org/10.1111/j.1365-2958.2011.07668.x>.
10. Sauviac L, Bruand C. 2014. A putative bifunctional histidine kinase/phosphatase of the HWE family exerts positive and negative control on the *Sinorhizobium meliloti* general stress response. *J Bacteriol* 196:2526–2535. <http://dx.doi.org/10.1128/JB.01623-14>.
11. Kaczmarczyk A, Vorholt JA, Francez-Charlot A. 2013. Cumate-inducible gene expression system for sphingomonads and other alphaproteobacteria. *Appl Environ Microbiol* 79:6795–6802. <http://dx.doi.org/10.1128/AEM.02296-13>.
12. Ulrich LE, Zhulin IB. 2010. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Res* 38:D401–D407. <http://dx.doi.org/10.1093/nar/gkp940>.
13. Zhulin IB, Nikolskaya AN, Galperin MY. 2003. Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. *J Bacteriol* 185:285–294. <http://dx.doi.org/10.1128/JB.185.1.285-294.2003>.
14. Sambrook J, Russell D. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. Kaczmarczyk A, Vorholt JA, Francez-Charlot A. 2012. Markerless gene deletion system for sphingomonads. *Appl Environ Microbiol* 78:3774–3777. <http://dx.doi.org/10.1128/AEM.07347-11>.
16. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T. 2014. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42:W252–W258. <http://dx.doi.org/10.1093/nar/gku340>.
18. Krissinel E, Henrick K. 2004. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D Biol Crystallogr* 60:2256–2268. <http://dx.doi.org/10.1107/S0907444904026460>.
19. Yeh JI, Biemann HP, Prive GG, Pandit J, Koshland DE, Jr, Kim SH. 1996. High-resolution structures of the ligand binding domain of the wild-type bacterial aspartate receptor. *J Mol Biol* 262:186–201. <http://dx.doi.org/10.1006/jmbi.1996.0507>.
20. Gaidenko TA, Bie X, Baldwin EP, Price CW. 2011. Substitutions in the presumed sensing domain of the *Bacillus subtilis* stressosome affect its basal output but not response to environmental signals. *J Bacteriol* 193:3588–3597. <http://dx.doi.org/10.1128/JB.00060-11>.
21. Gaidenko TA, Bie X, Baldwin EP, Price CW. 2012. Two surfaces of a conserved interdomain linker differentially affect output from the RST sensing module of the *Bacillus subtilis* stressosome. *J Bacteriol* 194:3913–3921. <http://dx.doi.org/10.1128/JB.00583-12>.
22. Kaczmarczyk A, Vorholt JA, Francez-Charlot A. 2014. Synthetic vanilate-regulated promoter for graded gene expression in *Sphingomonas*. *Sci Rep* 4:6453. <http://dx.doi.org/10.1038/srep06453>.
23. Klose KE, Weiss DS, Kustu S. 1993. Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. *J Mol Biol* 232:67–78. <http://dx.doi.org/10.1006/jmbi.1993.1370>.
24. Stock AM, Robinson VL, Goudreau PN. 2000. Two-component signal transduction. *Annu Rev Biochem* 69:183–215. <http://dx.doi.org/10.1146/annurev.biochem.69.1.183>.
25. Forst S, Inouye M. 1988. Environmentally regulated gene expression for membrane proteins in *Escherichia coli*. *Annu Rev Cell Biol* 4:21–42. <http://dx.doi.org/10.1146/annurev.cb.04.110188.000321>.
26. Hall MN, Silhavy TJ. 1981. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. *Annu Rev Genet* 15:91–142. <http://dx.doi.org/10.1146/annurev.ge.15.120181.000515>.
27. Mizuno T, Mizushima S. 1990. Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecu-

- lar basis for the osmotic regulation of the porin genes. *Mol Microbiol* 4:1077–1082. <http://dx.doi.org/10.1111/j.1365-2958.1990.tb00681.x>.
28. Pratt LA, Silhavy TJ. 1995. Porin regulon of *Escherichia coli*, p 105–127. In Hoch JA, Silhavy TJ (ed), Two-component signal transduction. ASM Press, Washington, DC.
 29. Galperin MY. 2006. Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J Bacteriol* 188:4169–4182. <http://dx.doi.org/10.1128/JB.01887-05>.
 30. 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 U S A* 103:13186–13191. <http://dx.doi.org/10.1073/pnas.0603530103>.
 31. Davis SJ, Vener AV, Vierstra RD. 1999. Bacteriophytochromes: phytochrome-like photoreceptors from nonphotosynthetic eubacteria. *Science* 286:2517–2520. <http://dx.doi.org/10.1126/science.286.5449.2517>.
 32. Wojnowska M, Yan J, Sivalingam GN, Cryar A, Gor J, Thalassinis K, Djordjevic S. 2013. Autophosphorylation activity of a soluble hexameric histidine kinase correlates with the shift in protein conformational equilibrium. *Chem Biol* 20:1411–1420. <http://dx.doi.org/10.1016/j.chembiol.2013.09.008>.
 33. Berleman JE, Bauer CE. 2005. Involvement of a Che-like signal transduction cascade in regulating cyst cell development in *Rhodospirillum centenum*. *Mol Microbiol* 56:1457–1466. <http://dx.doi.org/10.1111/j.1365-2958.2005.04646.x>.
 34. He K, Marden JN, Quardokus EM, Bauer CE. 2013. Phosphate flow between hybrid histidine kinases CheA(3) and CheS(3) controls *Rhodospirillum centenum* cyst formation. *PLoS Genet* 9:e1004002. <http://dx.doi.org/10.1371/journal.pgen.1004002>.
 35. Aguilar PS, Hernandez-Arriaga AM, Cybulski LE, Erazo AC, de Mendoza D. 2001. Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* 20:1681–1691. <http://dx.doi.org/10.1093/emboj/20.7.1681>.
 36. Cybulski LE, Martin M, Mansilla MC, Fernandez A, de Mendoza D. 2010. Membrane thickness cue for cold sensing in a bacterium. *Curr Biol* 20:1539–1544. <http://dx.doi.org/10.1016/j.cub.2010.06.074>.
 37. Tajima H, Imada K, Sakuma M, Hattori F, Nara T, Kamo N, Homma M, Kawagishi I. 2011. Ligand specificity determined by differentially arranged common ligand-binding residues in bacterial amino acid chemoreceptors Tsr and Tar. *J Biol Chem* 286:42200–42210. <http://dx.doi.org/10.1074/jbc.M111.221887>.
 38. Bjorkman AM, Dunten P, Sandgren MO, Dwarakanath VN, Mowbray SL. 2001. Mutations that affect ligand binding to the *Escherichia coli* aspartate receptor: implications for transmembrane signaling. *J Biol Chem* 276:2808–2815. <http://dx.doi.org/10.1074/jbc.M009593200>.
 39. Gardina P, Conway C, Kossman M, Manson M. 1992. Aspartate and maltose-binding protein interact with adjacent sites in the Tar chemotactic signal transducer of *Escherichia coli*. *J Bacteriol* 174:1528–1536.
 40. Mowbray SL, Koshland DE, Jr. 1990. Mutations in the aspartate receptor of *Escherichia coli* which affect aspartate binding. *J Biol Chem* 265:15638–15643.
 41. Wolff C, Parkinson JS. 1988. Aspartate taxis mutants of the *Escherichia coli* tar chemoreceptor. *J Bacteriol* 170:4509–4515.
 42. Letunic I, Doerks T, Bork P. 2012. SMART 7: recent updates to the protein domain annotation resource. *Nucleic Acids Res* 40:D302–D305. <http://dx.doi.org/10.1093/nar/gkr931>.
 43. Innerebner G, Knief C, Vorholt JA. 2011. Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. *Appl Environ Microbiol* 77:3202–3210. <http://dx.doi.org/10.1128/AEM.00133-11>.
 44. Nallamsetty S, Austin BP, Penrose KJ, Waugh DS. 2005. Gateway vectors for the production of combinatorially-tagged His6-MBP fusion proteins in the cytoplasm and periplasm of *Escherichia coli*. *Protein Sci* 14:2964–2971. <http://dx.doi.org/10.1110/ps.051718605>.